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A simple soft lithographic route to fabrication of poly(ethylene glycol) microstructures for protein and cell patterning

Kahp Y. Suh[a], Jiehyun Seong[a], Ali Khademhosseini[b], Paul E. Laibinis[a], Robert Langer[a,b,*]

[a] Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA
[b] Division of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

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Abstract

We present a simple, direct soft lithographic method to fabricate poly(ethylene glycol) (PEG) microstructures for protein and cell patterning. This lithographic method involves a molding process in which a uniform PEG film is molded with a patterned polydimethylsiloxane stamp by means of capillary force. The patterned surfaces created by this method provide excellent resistance towards non-specific protein and cell adsorption. The patterned substrates consist of two regions: the molded PEG surface that acts as a resistant layer and the exposed substrate surface that promotes protein or cell adsorption. A notable finding here is that the substrate surface can be directly exposed during the molding process due to the ability to control the wetting properties of the polymer on the stamp, which is a key factor to patterning proteins and cells.

Keywords: Microfabrication; Patterning; Immobilization; Biosensors; Cross-linking

1. Introduction

Conventional methods for creating microscopic structures and patterns that can be defined as microfabrication technologies have been successfully applied to protein or cell patterning because they can provide the ability to spatially control protein or cell adhesion. Although optical lithography or photolithography is one of the most well-established techniques for microfabrication, its usage with cells and proteins is restricted by steps that must be carried out in harsh organic solvents [1]. A more biocompatible method can be found in the photolithographic patterning of poly(ethylene glycol) (PEG) hydrogels, which generally involves three steps: spin coating of a PEG solution onto a substrate, exposing gel precursor through a photomask, and then developing using solvents such as water, toluene, or supercritical CO_2 [2–4]. Using this process, PEG microstructures have been successfully applied to the fabrication of enzyme electrodes [2], pH sensitive MEMS devices [3], and optical sensors [4]. However, photolithography appears slightly complicated and expensive to be the mainstay for protein or cell patterning.

A possible advancement of microfabrication may be sought in soft lithography, the collective name for a set of lithographic techniques that involve the use of an elastomer such as polydimethylsiloxane (PDMS). To transfer solutions of biomolecules or modify surfaces to a negative relief of the pattern, a number of soft lithographic techniques have been used to date including microfluidic networking [5–7], micromolding in capillaries [5,8] and microcontact printing (μCP) [9–13]. Of these, self-assembled monolayers (SAMs) terminated with PEG chains have been extensively used in μCP to immobilize biological species such as proteins or cells. This technique involves selective modification of surfaces with a non-biofouling agent like PEG [14,15], allowing a basis for the creation of patterned surfaces.

There also have been a number of other methods to generate patterns of proteins and cells on surfaces. These methods include membrane-based lift-off [16], polymer templating using μCP [17–20], manipulations of surface charge [21], hydrophilicity [21], and topography [22].

*Corresponding author. Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA.
E-mail address: rlanger@mit.edu (R. Langer).

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Although μCP is relatively simple and versatile for micropatterning, it cannot provide control over the topographical of a surface. To obtain topographical patterns, microfluidic networking or micromolding in capillaries can be used. However, these techniques are limited in that the mold should have an inner network structure and feature sizes below 1 μm have been difficult to realize [23]. Furthermore, there essentially has been no soft lithographic technique to pattern PEG hydrogels directly on the surface.

Here, we present an addition to these emerging patterning techniques in the form of capillary force lithography [24,25] based on the capillarity and wettability of the polymer within the stamp. In comparison to μCP, the molded PEG structure acts as a physical and biological barrier for the adhesion of proteins and cells. Such a barrier shows a unique regulation of surface patterning. Furthermore, capillary force lithography provides a general platform for patterning a broad range of materials since it can be applied to substrates such as glass, silicon, silicon dioxide and polymers. As a result, it may be a valuable tool for fabricating protein chips and high-throughput cell screening devices as feature sizes can be easily controlled ($\sim$500 nm to $\sim$500 μm) on a large area with suitably prepared PDMS molds.

2. Materials and methods

2.1. PDMS stamp fabrication

PDMS stamps were fabricated by casting PDMS (Sylgard 184 Silicon elastomer, Essex Chemical) against a complementary relief structure that was prepared by photolithographic method. To cure, a 1:10 ratio of the curing agent and the pre-polymer were mixed and incubated at 70°C for 1 h. The PDMS mold was then peeled from the silicon wafer and cut prior to use. The stamps had protruding (positive) features with the lateral dimension ranging from 10 to 500 μm.

2.2. Patterning of PEG films

Silicon and glass were used as the substrate. Prior to spin coating, substrates were cleaned by rinsing with acetone and ethanol several times to remove excess organic molecules and dried in nitrogen. Four different concentrations of PEG dimethacrylate (PEG-DMA) methanol solutions (20, 50, 80, and 100 wt% or pure polymer) were prepared. 1 wt% of the UV initiator (2,2-dimethoxy-2-phenylacetophenone, Aldrich) with respect to the amount of polymer was added in the solutions. The solution of PEG-DMA was then spin coated (Model CB 15, Headaway Research, Inc.) onto a substrate at 3000 rpm for 10 s. The film thickness ranged from 300 nm to 2 μm as measured by ellipsometry. The patterned PDMS stamp was carefully placed onto the surface to make conformal contact [25] and the sample was placed under a 365 nm, 15 mW/cm^2 low-power Black-light inspection lamp (ELC-251, Electro-Lite Corp.) for cross-linking.

2.3. Protein adsorption

Fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA) and fibronectin (FN) (Sigma) were dissolved in 10 mM phosphate buffer saline (PBS) (Sigma) solution (pH = 7.4; 10 mM sodium phosphate buffer, 2.7 mM KCl, and 137 mM NaCl) at concentrations of 250 and 100 μg/ml, respectively. A few drops of the protein solution were evenly distributed onto the patterned substrates and stored at room temperature for 30 min. The patterned substrates were rinsed with PBS solution and water, and blown dry in a stream of nitrogen and then directly analyzed under a fluorescent microscope (Axiovert 200, Zeiss).

2.4. Cell cultures

NIH-3T3 murine embryonic fibroblasts (Advanced Type Culture Collection) were maintained in Dulbecco's modified eagle medium (Gibco Invitrogen Corp.) supplemented with 10% fetal bovine serum (Gibco Invitrogen Corp.) at 37°C and 5% CO_2 environment. Once the cells were confluent, they were trypsinized (0.25% in EDTA, Sigma) and passaged at a 1:5 subculture ratio. To pattern FN onto PEG patterned or control surfaces, glasses were treated with 100 μg/ml of FN in PBS for 20 min. The surfaces were then washed with PBS to remove excess solution from the surfaces. NIH-3T3 cells suspended in medium at a concentration of 1×10^6 cells/ml were then plated on to the surfaces. To analyze, glass slides were rinsed with PBS to remove non-adhered cells from the non-adhered regions.

3. Results and discussion

To fabricate a PEG film on a substrate and prevent the immobilized PEG from dissolving in water, we used a low molecular weight PEG-DMA ($M_w = 330$) because it enables a high cross-linking density. Our method is based on capillary force lithography that was reported previously (Fig. 1) [24,25]. When a polymer film is mobile (i.e., above its glass transition temperature) and is in conformal contact with a patterned PDMS stamp, capillarity forces the polymer into the void space of the stamp, resulting in a negative replica of the stamp. When a hydrophobic polymer is used such as polystyrene or a poly(styrene–butadiene–styrene) copolymer, the polymer partially wets the hydrophobic PDMS wall

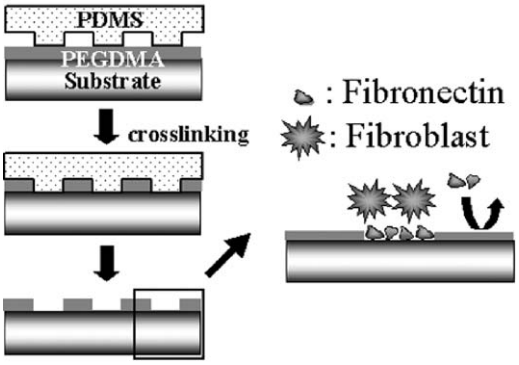

Fig. 1. Schematic diagram of the experimental procedure. Initially, a uniform PEG-DMA film is prepared by spin coating and then molded by capillary force lithography [24,25]. This pattern is utilized for subsequent protein and cell patterning using PEG-DMA as the blocking layer.

(acute contact angle), thus rising into the void space [24]. On the other hand, capillary rise will be depressed within a void space if the contact angle is larger than 90° (capillary depression). In capillary force lithography, the polymer in contact with the stamp gets depleted during the capillary rise and finally the substrate surface becomes exposed as shown in Fig. 1. As expected, this is only possible when the polymer is relatively thin with respect to the mold's step height. The resulting patterned substrates consist of two regions: the molded PEG-DMA surface that acts as a resistant layer and the exposed substrate surface that promotes protein or cell adsorption. Contact angle measurements of PEG-DMA on a fresh PDMS stamp (~65°) show that PEG-DMA is slightly hydrophilic and mobile on the PDMS surface such that the substrate surface can be easily exposed in comparison to hydrophobic polystyrene [24].

An alternative method to provide polymer mobility is to directly place a PDMS stamp onto a wet polymer surface. This technique generally consists of three steps: placing a patterned PDMS stamp on the surface of a spin-coated polymer film, allowing the stamp to absorb solvent, and then letting the stamp and the substrate remain undisturbed for a period of time. The microstructure thus formed shows high pattern fidelity without distortions or defects [25]. For PEG-DMA polymer, it turns out that the latter method is more appropriate to ensure pattern fidelity over large areas since dewetting patterns were observed with increasing temperature above the PEG-DMA's melting temperature (~50°C) probably due to the large contact angle. Therefore, we decided to use solvent instead of temperature to facilitate the movement of PEG-DMA.

In our experiment, a PDMS stamp was directly placed onto the polymer surface after spin coating for only 10 s to make a wet film (i.e., PEG + methanol), forming a conformal contact with the surface. As the solvent evaporates, PEG-DMA in contact with the protruding PDMS features spontaneously recedes and moves into the void space of the PDMS mold by means of capillary action. According to the previous report [25], this capillary action is completed within a few minutes. Because of the hydrophilic nature of PEG-DMA, it is expected that the substrate surface would be easily exposed on a hydrophobic substrate such as a polymer or clean silicon wafer. In this case, the repelling forces at the PEG-DMA/substrate interface facilitate the mass transfer, squeezing the film under the contact region into adjacent regions. However, it was observed that relatively hydrophilic substrate surfaces such as silicon dioxide and glass could also be exposed as confirmed by optical microscopy and atomic force microscopy (AFM), which indicates that solvent evaporation appears to play a crucial role in mass transfer. No dewetting takes place in spite of the large contact angle because the polymer is confined within the PDMS stamp and the mobility of the polymer is not sufficient to give rise to dewetting. Once the solvent is fully evaporated, the cured PEG layer acts as a barrier or confined wall for adsorbed proteins or cells. The aspect ratio of barriers could be controlled using different film thicknesses for a given feature size of the PDMS stamp.

Fig. 2 shows typical AFM images of a polymer microstructure (10 μm boxes) that was fabricated on a silicon wafer when a 50% solution was used for molding. As seen from the figure, the topographical features are well defined with high pattern fidelity (Figs. 2(a), (c) and (d)). A good contrast in the phase image in Fig. 2(b) represents two different kinds of interactions of the AFM tip with the surface, thus indicating that the substrate surface is completely exposed. The step height of the mold was 500 nm, which is nearly the same as the height from bottom to top of the cross-sectional image in Fig. 2(d). If the concentration of the polymer solution decreases, the topographical height also decreases, ultimately leading to polymer islands rather than a flat film within the void region when the concentration is lower than a certain critical value (mass depletion). Although mass depletion is mainly responsible for the morphology, the competition between capillarity and surface tension also play a role in determining the particular morphology, which makes the molding process complicated to quantitatively describe [26]. Furthermore, the topography is also dependent on other driving forces such as alterations to pressure, viscosity, and surface properties of the various components of the system. In Fig. 2(d), a meniscus is observed within the void space of the stamp, which is attributed to the contact angle at the PEG-DMA/PDMS/air interface.

To monitor the curing of PEG-DMA films, we carried out Fourier transform infrared spectroscopy as a function of UV exposure time. Fig. 3 shows the transition of two peaks that are assigned to C = O and C = C stretch, respectively. The IR spectrum prior to

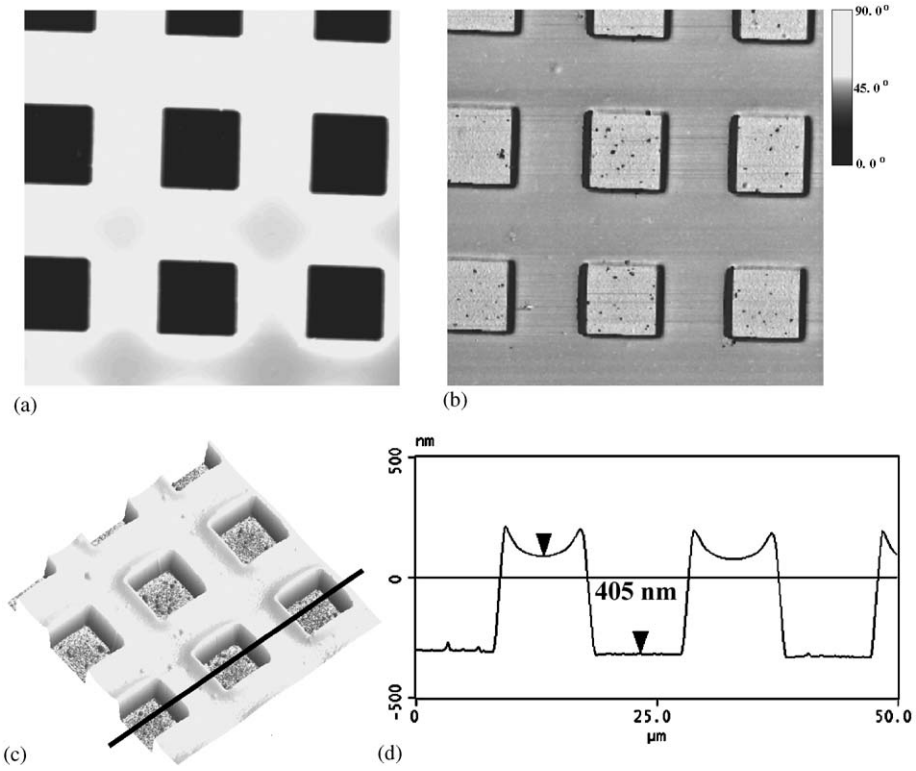

Fig. 2. Two-dimensional height (a) and phase (b) images of a molded PEG-DMA film for 10 μm box pattern. A sharp contrast in the phase image indicates that the substrate surface is completely exposed. (c) A three-dimensional presentation of (a) and (d) a cross-sectional view of the line in (c). The scan size is $50 \times 50\,\mu m^2$.

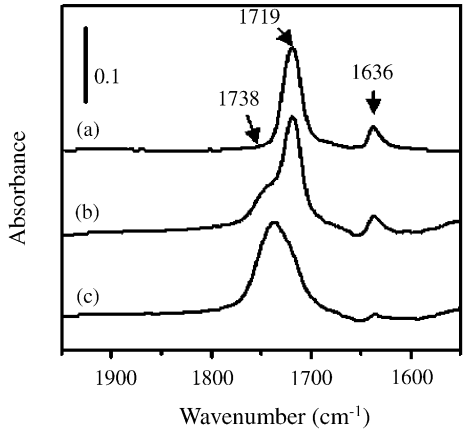

Fig. 3. FT-IR spectra of a cured PEG-DMA ($M_w = 300$) film at different UV exposure times: (a) before UV exposure, (b) after 2 h, and (c) after 5 h.

UV exposure features sharp bands at 1719 and $1636\,cm^{-1}$, which are characteristic of conjugated ester and terminal C = C of methacrylates, respectively (Fig. 3(a)). After exposure for 2 h (Fig. 3(b)), some of the methacrylates (i.e., conjugated esters) reacted and a peak at $1738\,cm^{-1}$, which corresponds to the C = O stretch of a saturated aliphatic ester, started to appear. In addition, the peaks of conjugated ester (1719 and $1636\,cm^{-1}$) decreased compared to their initial intensities, which indicates the presence of cross-linked methacrylate chains. The degree of C = C conversion after 2 h illumination is about 63% by taking into account the ratio of two peaks around $1636\,cm^{-1}$ in Figs. 3(a) and (b). A careful examination of surface morphology at this conversion shows that the film absorbs a small amount of water and swells in PBS such that bubbles are observed after immersion for 24 h. However, this bubbled surface also turned out to be effective for selective protein and cell adsorption. If the cross-linking density is too low (when exposure time is less than 1 h), however, the film dissolves or delaminates from the substrate due to the swelling stress at the PEG-DMA/substrate interface. After 5 h exposure, the peak of C = O stretching completely shifted to $1738\,cm^{-1}$ and that of C = C ($1636\,cm^{-1}$) is substantially reduced, which means that the film is completely cross-linked.

To examine the effectiveness of the PEG pattern and surfaces to reduce protein adsorption, we prepared FITC-BSA and FN (not fluorescein labeled) solutions dissolved in PBS. As PEG surfaces are well known for resisting protein adsorption [27], BSA and FN are expected to adsorb only on the exposed glass surface. As shown in Fig. 4, we observed a spatially well-defined fluorescent image from FITC-BSA for 10 μm lines (Fig. 4(a)) and a neat patterned structure from FN for 10 μm boxes (Fig. 4(b)). This indicates that the cured PEG-DMA film also shows protein resistance as with PEG

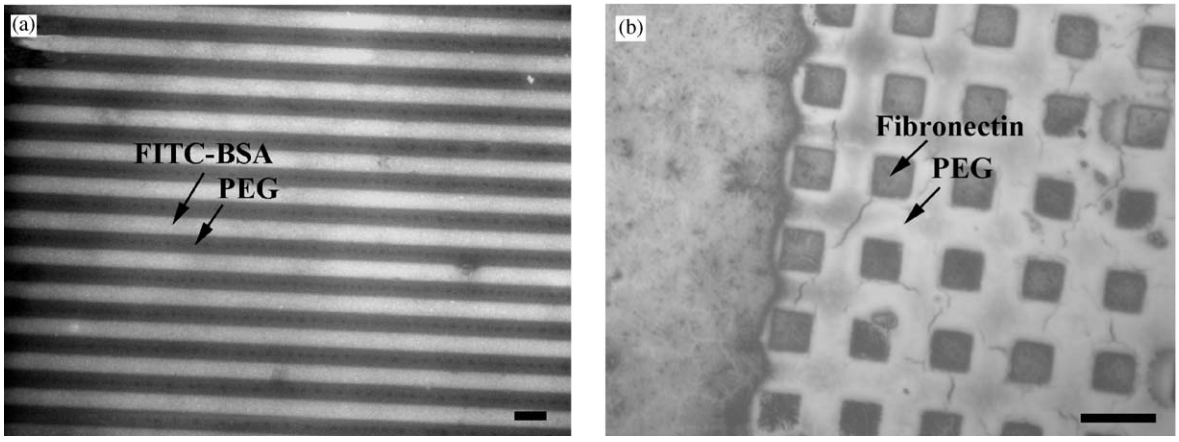

Fig. 4. (a) A fluorescent micrograph of FITC-BSA that is selectively adsorbed on the 10 μm lines of PEG-DMA surface. Sharp contrasts are observed. The scale bar indicates 20 μm. (b) An optical micrograph of FN (not fluorescein labeled) that is selectively adsorbed on the 10 μm boxes of PEG-DMA surface. One can observe a drastic difference between the polymer and bare surface with the aid of the boundary line. The scale bar indicates 20 μm.

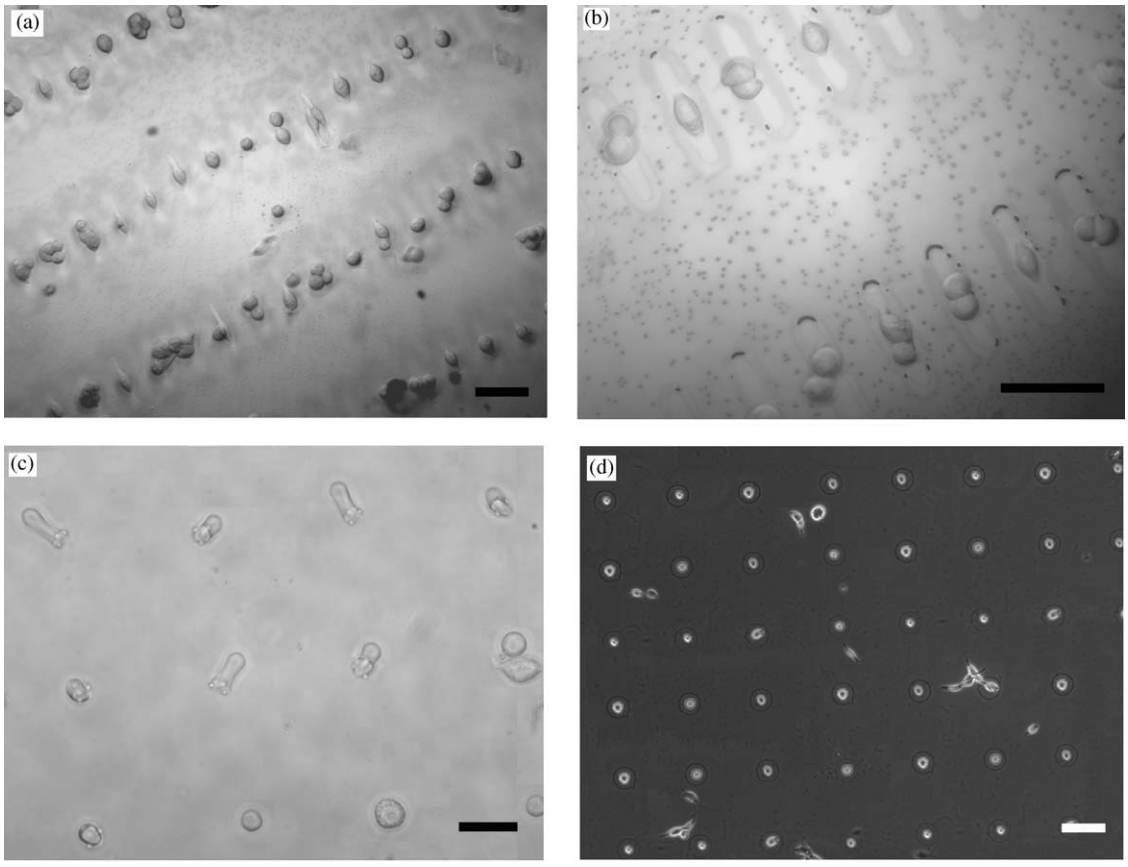

Fig. 5. Optical micrographs of NIH-3T3 cells deposited on (a) $15 \times 75\,\mu m^2$ ovals on silicon wafer, (b) same as (a) (magnified), (c) 25 μm circles on silicon wafer, and (d) 25 μm circles on glass. As shown in the figure, cells are confined in PEG-DMA barriers with numbers strictly controlled by the feature sizes. The scale bar indicates 50 μm.

terminated SAM molecule [27], which can be readily understood in that the molecular backbone is maintained regardless of cross-linking and its density.

As FN is known to mediate cell adsorption [16,27], we can prepare arrays of cell patterns with the aid of adsorbed FN. For this purpose, we prepared NIH-3T3 murine embryonic fibroblasts in PBS at a concentration of 1×10^6 cells/ml. Prior to patterning, PEG patterns prepared on glass and silicon wafers were treated with FN for 20 min. NIH-3T3 cells suspended in medium were then plated onto the surfaces and the cell cultures were analyzed at various times. Fig. 5 shows typical cell

images that can be obtained with patterned PEG-DMA surfaces when incubated for 4 h. As expected, cells appear to deposit only on the exposed glass or silicon wafer, not on a PEG surface. The figure also shows that the number of cells on the surface can be controlled by varying the relative size of the cell and that of the barrier. The initial cell size of NIH-3T3 is estimated to be about 10 μm. Once the cell is immobilized on the surface, it spreads and grows in size. One–three cells can be deposited within the barriers for $15 \times 75\,\mu m^2$ ovals (Figs. 5(a) and (b)) and a single cell for 25 μm diameter circles (Figs. 5(c) and 3(d)). Thus, cells cannot be deposited on the surface if the pattern size is less than 10 μm. Although cell patterning for larger feature sizes is under study, it is expected that the feature size can strictly control the number of adsorbed cells, which could make it possible to screen cell adsorption in a simple and convenient manner. In addition, the PEG microstructures form a physical barrier, which further prevents cell spreading in comparison to techniques in which only the substrate surface is modified. Although a PDMS membrane has been used for physical confinement [16], its application is limited in that the pattern size is typically on the order of a hundred micrometers and PDMS is not inert for the adhesion of cells. In this regard, it would be intriguing to examine the spreading behavior of individual cells in the presence of narrow, high aspect-ratio PEG walls, which could provide valuable information on cell culture in a confined, non-sticky geometry.

4. Conclusion

We have developed capillary force lithography as a tool for general-purpose lithography for protein and cell patterning. By introducing PEG-DMA as the patterned polymer, spatially well-defined images of selective protein and cell adsorption are observed on a large area, which could open new pathways for fabricating protein chips and high-throughput cell screening devices. The number of cells in a given barrier can be controlled depending on the feature size. As the demand for strict control of cell position and function is increasing for applications ranging from cellular to tissue engineering and biosensors [28], the technique presented here could be increasingly useful for such purposes.

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