Improved single-cell culture achieved using micromolding in capillaries technology coupled with poly (HEMA)

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(Received 29 December 2014; accepted 2 July 2015; published online 24 July 2015)

Cell studies at the single-cell level are becoming more and more critical for understanding the complex biological processes. Here, we present an optimization study investigating the positioning of single cells using micromolding in capillaries technology coupled with the cytophobic biomaterial poly (2-hydroxyethyl methacrylate) (poly (HEMA)). As a cytophobic biomaterial, poly (HEMA) was used to inhibit cells, whereas the glass was used as the substrate to provide a cell adhesive background. The poly (HEMA) chemical barrier was obtained using micromolding in capillaries, and the microchannel networks used for capillarity were easily achieved by reversibly bonding the polydimethylsiloxane mold and the glass. Finally, discrete cell adhesion regions were presented on the glass surface. This method is facile and low cost, and the reagents are commercially available. We validated the cytophobic abilities of the poly (HEMA), optimized the channel parameters for higher quality and more stable poly (HEMA) patterns by investigating the effects of changing the aspect ratio and the width of the microchannel on the poly (HEMA) grid pattern, and improved the single-cell occupancy by optimizing the dimensions of the cell adhesion regions. © 2015 AIP Publishing LLC.

[<http://dx.doi.org/10.1063/1.4926807>]

I. INTRODUCTION

Single-cell culture has enormous potential for a range of applications, including drug testing, toxicology studies, cell biology research, and tissue engineering.^{1–3} Specific technologies are required to confine single cells to particular locations on a substrate. Many strategies exist for controlling the position of cells, and these techniques fall into three categories: (i) seeding cells on a chemically patterned surface with regions having different degrees of adhesiveness for the cells, (ii) seeding cells on a topographically patterned surface, and (iii) directed delivery of cells to discrete regions of a substrate.⁴

The critical step in the chemical method is the formation of alternating patterns of permissive or non-permissive surface regions, which, respectively, promote and suppress cell growth using cytophilic and cytophobic chemicals.⁵ A number of research groups have developed various methods to pattern cytophilic and cytophobic chemicals on a substrate. Such methods typically combine microfabrication, chemical surface modification, and material processing.^{6,7} Among these methods, microcontact printing (μ CP) is the most frequently used.^{8–10} The chemical materials that are typically used include ethylene glycol-functionalized alkanethiols, adhesive proteins (i.e., extracellular matrix), or their interactive peptide derivatives. However,

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alkanethiols are not easily available, high-quality gold-coated surfaces are both expensive and incompatible with some applications,¹¹ and the adhesive proteins that can be used to bind the cells to the surface can be easily degraded; it is also difficult to control the transfer efficiency when delivering molecules to substrates using μ CP.¹² The precise control of surface ligand densities is important for studying cell–matrix interactions. An alternative approach called micro-molding in capillaries (MIMIC) has also been used to achieve chemical surface patterning. In summary, a poly (dimethyl-siloxane) (PDMS) mold containing open microchannels is inverted on the glass to form closed capillaries, and a chemical solution is then introduced into the capillaries under the influence of capillary forces to functionalize the exposed regions.¹³ This method has the advantage of being compatible with many types of surfaces. Furthermore, the MIMIC technique can be more readily applied to control the thickness of chemical materials, compared with the μ CP technique.⁶ However, MIMIC restricts the complexity, types, and size of the patterns that can be generated and is typically used to create continuous patterns (and is thus unable to realize single-cell culture).¹⁴ A new method has been developed in which the combination of MIMIC technology and cytophobic material is used to precisely control the positioning of cells.^{15,16} Using this method, latticed and discrete cytophobic regions can be obtained on a glass surface, and these regions can restrict single cells on the glass surface. The use of this type of patterning substrate to achieve single-cell culture has been reported;^{17,18} however, to the best of our knowledge, the pattern quality and the pattern parameters involved in limiting single cells to the restricted areas have not been fully characterized.

Here, we present an optimization study investigating the realization of single-cell occupancy in large arrays and an evaluation of the performance of the cytophobic pattern. With this method, we determined the pattern parameters that maximized the single-cell occupancy.

II. MATERIALS AND METHODS

A. Improved MIMIC for single-cell culture

Figure 1(a) shows a schematic of the traditional MIMIC technique used to achieve cell micropatterning. The capillary microchannels are formed between the PDMS mold and the cytophobic substrate. Chemical materials then fill the microchannels under capillary action and form alternating micropatterns that, respectively, promote and suppress cell growth. The chemical materials used are typically cytophilic, so that the attachment regions for the cells are continuous, which is clearly unsuitable for single-cell culture. To overcome such limitations, we developed an improved MIMIC technology for single-cell culture, as illustrated in Fig. 1(b). Instead of a cytophilic material, we used a cytophobic material. The substrate was cytophilic, and microchannels were designed to form a grid pattern. As a result, discrete cell adhesion regions surrounded by cytophobic were achieved. The results showed that the combination of the MIMIC technique with a cytophobic material could be used for single-cell culture.

FIG. 1. (a) The traditional MIMIC technique used for cell patterning. (b) Improved MIMIC technique for single-cell culture.

B. Assessment of the stability of the chemical pattern

Poly (2-hydroxyethyl methacrylate) (poly (HEMA), Sigma-Aldrich, USA) was used as the cytophobic material to construct the patterning surface.^{19,20} The ability of the patterning substrate to restrict single cells to confined areas depended on the quality of the poly (HEMA) grid pattern. The grid pattern quality relied primarily on the integrity of the chemical pattern. If the nonspecific adsorption of proteins from the medium or secreted from cells obscures the poly-(HEMA), the integrity of the pattern will be damaged, and this will lead to the failure of the single-cell culture.

We designed the protein adsorption trial according to that presented by Cheng *et al.*²¹ Fluorescein isothiocyanate (FITC)-labeled bovine serum albumin (BSA) was used to evaluate the nonspecific protein adsorption on the poly (HEMA). The samples were covered with an FITC-BSA solution with an area density of 100 mg/cm^2 and were incubated for 1 h at room temperature in a dark space. The samples were then washed twice with deionized water, and allowed to dry in a clean laboratory environment before they were observed using fluorescence microscopy. The amount of adsorbed FITC-BSA was determined from the intensity of the green color, which was measured from the fluorescence micrographs. A piece of glass coated with only tris-(hydroxyl-methyl) methylamine (FITC-BSA solvent) was used as black reference, and a piece of glass coated with FITC-BSA that was not washed before observations were made, which was used as bright reference.

C. Fabrication of the patterning surface

Figure 2 illustrates the steps used to fabricate the poly (HEMA) grid pattern.¹⁶ First, the silicon master was prepared using traditional photolithography and inductively coupled plasma (ICP) technology (Fig. 2(a)). Briefly, 4-in. silicon wafers (Junhe Electronic Material Inc., Shanghai, China) were used as substrates for the master fabrication. The wafers were baked in an oxidation furnace at 800°C for 20 min to remove any adsorbed moisture. Photoresist EPG 533 (Everlight Chemical Corp., Taiwan) was then spread for 15 s at 500 rpm, and then spun for 60 s at 4000 rpm. Immediately after spinning, to evaporate the solvent, each wafer was placed on a 90°C hot plate for 1 min. The wafers were then exposed to an ultraviolet dose of 7.5 mW/cm^2 . The substrates were immersed in a 5% KOH solution for 40 s to remove the unexposed portions of the photoresist. After washing in deionized water for 5–10 min and drying in N_2 , the wafers were placed on a 90°C hot plate for 3 min. The exposed silicon was

FIG. 2. The process used to fabricate the poly (HEMA) grid pattern on the coverslip.

deep reactive ion etched (ICP-ASE, UK Investment Kewlspots Co., Ltd.) to form the silicon master. The master represented the negative (inverse) structure of the desired PDMS mold. The replica molding technique was then used to fabricate the PDMS molds (Fig. 2(b)). A typical PDMS sample (Sylgard 184, Dow Corning, USA) consisted of the polymer base and curing agent mixed at a ratio of 10:1. The mixture was cast on the silicon master and cured in an oven at 80 °C for 60 min. After curing, the PDMS mold was removed from the master (Fig. 2(c)). Next, the PDMS mold was cut into 10 × 10 mm blocks and bonded to the coverslip to form the microchannel networks used to achieve the capillarity. An ethanol solution of poly-(HEMA) was placed at the open ends of the microchannel networks and was allowed to spontaneously fill the void spaces under capillary action (Fig. 2(d)). Finally, a poly (HEMA) grid pattern was obtained on the coverslip after the ethanol had evaporated thoroughly (Fig. 2(e)). The concentration of the ethanol solution of poly (HEMA) was 50 mg/ml. The thickness of the poly-(HEMA) was measured using a Profile System (Ambios XP-2, USA).

Prior to bonding, the coverslips (22 × 22 mm) were cleaned to ensure that they could be used for the cell culture. Briefly, the coverslips were first cleaned using a surfactant, then rinsed with deionized water, and dried in an oven at 80 °C. They were then soaked in potassium dichromate overnight (or for at least 6 h), washed using abundant deionized water, and thoroughly blow dried with N₂.

D. Design of the cell culture substrate structure

Here, the poly (HEMA)-modified patterning glass surface was used to precisely control the positioning of the cells. Under the influence of the capillary action of the capillary microchannels formed between the PDMS mold and the glass substrate, the poly (HEMA) filled the microchannels to form a poly (HEMA) grid pattern. The patterning surface of the cell culture substrate therefore included alternating patterns of non-permissive (cytophobic) and permissive (cytophilic) surface regions that, respectively, suppressed and promoted cell growth; these regions were provided by the poly (HEMA) grid pattern and the cell adhesion regions, respectively. The dimensions of the poly (HEMA) grid pattern determined the stability of the pattern, while the dimensions of the cell adhesion regions determined the confining effects for single cells.

The cell adhesion region dimension was first fixed at 50 μm, while the channel (grid) width was set at 20, 40, or 60 μm. The channel (grid) width was then fixed at 20 μm, while the cell adhesion region dimension was set at 20, 60, or 80 μm (Table I).

E. Cell culture

L929 fibroblast cells were cultured in Dulbecco's modified Eagle medium (DMEM, Hyclone) supplemented with 10% fetal bovine serum (Hyclone) and penicillin–streptomycin. Trypsin (0.25%) was used to detach the cells from the culture flasks, and the cells were centrifuged at 1000 rpm for 7 min. Before cell seeding, the poly (HEMA) grid pattern on the coverslips was immersed in sterilized phosphate-buffered saline for 24 h. Next, fibroblastoma cells were loaded onto the micropatterned surfaces in 12-well culture plates (Nunc) at a cell seeding

TABLE I. Dimensions of the poly (HEMA) grid pattern and the cell adhesion region (μm).

Design No.	Cell adhesion region width	Poly (HEMA) grid width
1	a = 50	b = 20
2	a = 50	b = 40
3	a = 50	b = 60
4	a = 20	b = 20
5	a = 60	b = 20
6	a = 80	b = 20

density of 3×10^4 cells/cm². The cells were allowed to grow at 37 °C in a humidified 5% CO₂ incubator for 24 h.

F. Statistical analysis

For all of the experiments shown here, at least three non-overlapping pictures were taken of each region, covering about half of the entire region, and the number of cells in each micro-structure was counted.

III. RESULTS AND DISCUSSION

A. Prevention of non-specific protein binding

The adsorption of FITC–BSA protein on the poly (HEMA) surfaces of different thickness could be interpreted in light of the measured fluorescence intensity, as illustrated in Fig. 3. The color intensities of the black and bright reference surfaces were set equal to 0% and 100%, respectively. With increases in the thickness of the poly (HEMA), the fluorescence intensity decreased. This showed that the likelihood of proteins from the media or secreted from the cells obscuring the poly (HEMA) grid pattern in the cell culture process was decreased with increases in the thickness of the poly (HEMA). The cell adhesion is related to the proteins of the cell membrane and medium especially serum.²² Folkman and Moscona found that when the thickness was increased from 0.0035 μ m to 35 μ m, the adhesivity of the cells decreased correspondingly.²³ This result agreed well with our experimental data. However, no significant difference was observed in the cell-repelling performance of the poly (HEMA) when the thickness was within the range of 0.7–7.4 μ m; the poly (HEMA) could inhibit the non-specific binding of proteins even when the film was only 0.7 μ m thick.

B. Optimization of the microchannel size

The influence of the microchannel aspect ratio on the filling speed is illustrated in Figs. 4(a) and 4(b). The aspect ratio was varied to give values of 0.1, 0.2, and 0.5. However, the aspect ratio of the microchannel was not the key parameter determining the quality of the poly (HEMA) grid patterns when the aspect ratio was changed from 0.1 to 0.5 (Fig. 4(a)).

FIG. 3. Fluorescence intensity of FITC-labeled BSA protein adsorbed on poly (HEMA) grid patterns with different thicknesses.

FIG. 4. (a) Relationship between the filling time and the filling length for different microchannel aspect ratios. (b) Experimental results of filling the microchannel with ethanol (70 $\times$). The arrows show the flow direction. (c) The relationship between the filling time and the filling length for the same microchannel aspect ratio.

Corresponding experimental data indicated the same result (Fig. 4(b)). Taking into consideration both the fabrication cost and the filling speed, we chose 0.25 as the value for the channel aspect ratio for the subsequent experiments.

The influence of the microchannel width on the filling speed was investigated, and the results were shown in Fig. 4(c). When the aspect ratio was 0.25 and the width of microchannel was set at 20 μm , 40 μm , or 60 μm , the influence of changes in the microchannel width is limited; the quality of patterns showed no significant differences (Fig. 5). When the width was increased, the thickness of the poly (HEMA) grid pattern also increased (Table II). Because of the evaporation of the ethanol reagent and the low concentration of the solution, the poly-

FIG. 5. SEM images of poly(HEMA) grid patterns on glass. (a) $a = 50 \mu\text{m}$, $b = 20 \mu\text{m}$, (200 $\times$, 900 $\times$); (b) $a = 50 \mu\text{m}$, $b = 40 \mu\text{m}$, (200 $\times$, 700 $\times$); (c) $a = 50 \mu\text{m}$, $b = 60 \mu\text{m}$, (200 $\times$, 600 $\times$). The scale bars represent (a) 200 μm , 50 μm ; (b) 200 μm , 50 μm ; (c) 200 μm , 100 μm , respectively.

TABLE II. Thickness of the poly (HEMA) after the volatilization of the ethanol, with the same microchannel aspect ratio (μm).

Design No.	Design size of the silicon master	Aspect-ratio of grid channel	Poly (HEMA) grid pattern thickness
1	$a = 50,^a b = 20,^b h = 5^c$	0.25	0.700
2	$a = 50,^a b = 40,^b h = 10^c$	0.25	3.392
3	$a = 50,^a b = 60,^b h = 15^c$	0.25	6.364
4	$a = 50,^a b = 80,^b h = 20^c$	0.25	7.338

^a a is the cell adhesion region width.^b b is the poly (HEMA) grid width.^c h is the height of the microchannel.

(HEMA) grid width was much narrower than the channel width. The width of the poly-(HEMA) grid was typically 30%–40% of the channel width.

In view of the swelling of the poly (HEMA) immersed in the deionized water, we designed an experiment to investigate the relationship between the thickness of the poly (HEMA) and the stability of the microstructure. The images of the poly (HEMA) grid pattern after immersion in water for 0 h, 24 h, and 48 h are shown in Figs. 6(a)–6(c). When the thickness of the poly-(HEMA) was increased, the stability of the microstructure decreased. This might have been due to the weak combination of the poly (HEMA) with the glass substrate. To obtain a high-quality poly (HEMA) pattern, a width of $20\ \mu\text{m}$ and a height of $5\ \mu\text{m}$ were chosen as the optimal microchannel dimensions.

C. Optimization of the cell adhesion region dimensions

The dimensions of the cell adhesion regions determined the number of cells that was confined in the regions. Three different dimensions ($D = a \times a$; $a = 20\ \mu\text{m}$, $60\ \mu\text{m}$, and $80\ \mu\text{m}$) were studied. In accordance with the optimal values determined as described in Section III B, the microchannel width, height, and aspect ratio were $20\ \mu\text{m}$, $5\ \mu\text{m}$, and 0.25, respectively.

FIG. 6. Images of poly (HEMA) patterns after immersion tests. The magnification is $350\times$. The scale bar is $200\ \mu\text{m}$.

FIG. 7. Fluorescence micrographs of L929 cell patterning after 24 h of culture. (a) $a = 20\ \mu\text{m}$, $b = 20\ \mu\text{m}$; (b) $a = 60\ \mu\text{m}$, $b = 20\ \mu\text{m}$; (c) $a = 80\ \mu\text{m}$, $b = 20\ \mu\text{m}$.

Cells were loaded and cultured on these surfaces via random seeding under identical conditions. After incubation for 24 h, images of the patterned stained cells were collected simultaneously using the Hoechst 33258 channels of the fluorescence microscope. Figure 7 shows fluorescence micrographs of the L929 cells. When the width of the cell adhesion region was $20\ \mu\text{m}$, the single cell occupancy reached 43.4% in a 19×30 array. To quantify the cell occupancies of the aforementioned three different diameters, the number of cells in each cell adhesion region width was also counted (Fig. 8).

IV. CONCLUSION

Here, we have presented a simple but useful method of functionalizing a surface for single-cell culture by combining a cytophobic material with MIMIC technology. An ethanol solution of poly (HEMA) was patterned using microchannel networks without surface modification, and a patterned surface with defined cell “repellent/adherent” properties was thereby achieved on glass. Furthermore, the poly (HEMA) with certain thickness completely prevented non-specific protein binding, and the serum proteins in the culturing medium therefore did not bind to the poly (HEMA) grid pattern as the culturing day proceeded, and, finally, the cell could be confined in the defined area without any degradation in the pattern.

By optimizing the microchannel size, higher quality and more stable poly (HEMA) patterns were achieved, and a grid of $20\ \mu\text{m}$ was experimentally verified to be the most suitable for creating single L929 cell arrays. The poly (HEMA) micropatterns created using MIMIC as

FIG. 8. Distribution of the cell occupancies for different cell adhesion region widths.

described in this manuscript could be employed in cell biology to study specific cell physiological activity and could be applied for drug screening, clinical diagnostics, tissue engineering, and the development of cell-based sensors. In the future research, on the one hand, the rate of single-cell occupancy is an important aspect; on the other hand, the patterning method can be used to construct various graphics, including square, circular, and rhomb, which can be used in the control of cell morphology. Additionally, it can be used to investigate the cell-to-cell communication between cells.

ACKNOWLEDGMENTS

We thank Mr. Yanpeng Hao for drawing the flowcharts, and Mrs. Jianping Bai for directing cell experiments. This work was supported by the National Key Scientific Instrument and Equipment Development Project (2013YQ190467), 111 project (Grant No. B13044), and the National Natural Science Foundation of China (Grant Nos. 51375398, 50775188, and 51475384). All cell culture experiments were performed at the Fourth Military Medical University.

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