Fabrication of hexagonally packed cell culture substrates using droplet formation in a T-shaped microfluidic junction

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A method is here proposed to fabricate ordered hexagonally packed cell culture substrates with hexagonally arranged cell patterning areas. We generated photo-sensitive polymeric microdroplets in a T-shaped microfluidic junction by an immiscible liquid, and then solidified the collective self-assembled hexagonal droplet array to obtain the cell culture substrate, on which we took the grooves formed between the solidified droplets as the hexagonally arranged cell patterning areas. The most promising advantage of our method is that we can actively tune the droplet size by simply adopting different volumetric flow rates of the two immiscible fluids to form cell culture substrates with differently sized cell patterning areas. Besides, the examination results of the cell culture substrate's characteristics validate whether our method is capable of creating substrates with high spatial uniformity. To verify the cell patterning function of our cell culture substrates, we used the semi-adherent RAW cells to demonstrate the effectiveness of patterning of suspended/adherent cells before/after adhesion. Over 90% cell viability and cell patterning rate suggest that our method may be a promising approach for future applications of cell patterning on biochips. © 2013 American Institute of Physics. [<http://dx.doi.org/10.1063/1.4774315>]

I. INTRODUCTION

Due to the emerging study of cell biology, micro-scale cell patterning has attracted increasing attention.¹ The micro-scale cell patterning can be applied not only in the field of tissue engineering but also in cell-cell interaction,² drug screening,³ and biosensors.^{4,5}

Some methods for cell patterning that have been proposed mainly use forces to manipulate the cells, such as using the optical tweezers to control a single cell⁶ or using a microlens array as an optical tweezers array to manipulate multiple cells;⁷ the cells can also be patterned via acoustic tweezers based on the standing wave of sound;⁸ other methods include dielectrophoretic force,^{9,10} magnetic micropillar array,¹¹ micro magnetic concentrator devices,¹² or magnetic thin film array.¹³ These active methods are used mostly for the patterning of suspension cells. In contrast, the adherent cells are usually patterned by relatively passive and conventional methods which typically modify the material surface biophysically or chemically to define specific areas for cell adhesion, and these areas determine the way the cells are patterned depending on the interaction between the ligands on the material surface and the receptors on the cell surface. The approaches of defining the cell adhesion area include soft lithography,^{14,15} micro contact printing,^{16,17} microchannels,^{18,19} microwell arrays,^{20–22} microgroove arrays,^{5,23,24} porous structures,^{25–27} and 3D colloid arrays.²⁸

Much *in vitro* research has introduced microstructures as the contact guidance for cells. Cell behaviors including cell orientation, migration, proliferation, and cell adhesion morphology have been studied on microstructures with specific topographic features. In this study, we propose a fabrication method, which allows the control of the size of fabricated ordered

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hexagonally packed polymeric cell culture substrates. We dripped polymeric microdroplets by using an immiscible liquid in a T-junction microchannel,²⁹ and the main structure of the cell culture substrates was created by solidifying the self-assembled droplet array. The grooves between solidified droplets then served as the patterning area for the cells after surface modification. By this method, we can actively tune the dimensions of the cell culture substrate by simply adopting different volumetric flow rates of the two immiscible fluids in a microchannel, so it allows rapid experimentation. Hexagonal contact guidance provided by the cell culture substrates in this study can be useful for possible applications on orientation or transformation of fibroblast cells,^{30,31} cross-link of nerve cells,^{32,33} and formation of liver lobules.³⁴ Furthermore, the actively tunable feature of our method is very promising for the fabrication of variable-sized ordered substrates for cell culture.

II. EXPERIMENT DETAILS

A. Cell culture substrate fabrication

The fabrication process of the cell culture substrate included two major steps: the formation of the substrate body and the modification of the substrate surface. Figure 1 shows the process flow of the substrate body fabrication. The entire process was completed in a single polydimethylsiloxane (PDMS) microchannel with channel height $90\text{ }\mu\text{m}$. The PDMS microchannel was oxygen plasma bonded with a PDMS bottom plate instead of a glass plate used generally. The fabrication of the substrate body involved three steps: generation of the droplets, soft curing of the droplet material, and hard curing. In the first step, we used a T-shaped junction to create the microdroplets for constituting the substrate body. A dispersed phase fluid was injected from the lateral channel (with width $w_1 = 80\text{ }\mu\text{m}$) as the substrate body material, and a continuous phase fluid, which was immiscible to the dispersed phase fluid, was injected from the main channel (with width $w_2 = 100\text{ }\mu\text{m}$). The collision of these two fluids resulted in the dispersed droplets of the substrate body material. The substrate body material used in our experiment was tripropylene glycol diacrylate (TPGDA, Chembridge International Corp.), which is a photo-sensitive polymer material that solidifies after UV curing. The continuous phase fluid we used was water. In order to prevent the merging of the TPGDA droplets, 2% of span-80 (Sino-Japan Chemical) and 2% of sodium dodecyl sulfate (SDS) (Protech Technology Enterprise Co., Ltd.) were added to TPGDA liquid and water, respectively, as the surfactant agents. In principle, SDS was not necessary for stabilizing the emulsion. However, we found that the usage of SDS obviously made the emulsion stabilized for a longer time comparing to a case where only SPAN-80 was used. Besides, since both the material of the microchannel and bottom plate were PDMS, hydrophilic treatment with oxygen plasma was performed before the TPGDA was injected into the channel.³⁵ The purpose of hydrophilic treatment was to prevent the oil-based TPGDA from flowing along the wall of the main channel.

Once the TPGDA droplets were formed, we directed them into a channel section with expanded opening to slow down the flow, and at the same time, we applied a 50 W UV light

FIG. 1. Fabrication process of the cell culture substrate in a T-junction microfluidic channel.

(ADAC System™ Cure Spot 50) for 0.04 s to slightly solidify the droplets, which corresponds to the soft curing process (step 2) in Fig. 1. In this step, a thin skin formed over the droplet surface while the internal body remained liquid. This step was to prevent the deformation or merging of the TPGDA droplets upon collision before they were properly positioned and packed. After soft curing, the partially solidified TPGDA droplets were directed into a storage area (5 mm × 10 mm), and then self-assembled into hexagonal close packed array, during which the UV light remained applied but with longer duration (50 W for 8 s). This step is called hard curing, which was to completely solidify the droplets. The channels were then removed and the main body of the cell culture substrate was obtained. Note that the TPGDA droplets should not be completely solidified before they have accumulated, otherwise the solid spheres will not link together to form a robust platform.

The cell culture substrate we obtained so far was not yet suitable for cell patterning due to the material TPGDA possesses minor toxicity to the cells. Therefore, a biocompatibility treatment shown in Fig. 2 was introduced on the surface of the TPGDA array. PDMS was chosen as the biocompatible surface,³⁶ and diluted PDMS solution (PDMS:silicon oil (10 cs) = 1:2) was dripped onto the TPGDA array to completely cover the surface of the TPGDA array (see Figs. 2(a) and 2(b)), followed by baking of the PDMS covering (90 °C for 40 min) was performed to accomplish the substrate fabrication. The PDMS modification not only provided biocompatibility but also made the substrates less susceptible to dismemberment. Figure 2(c) shows the schematic of cell patterning on the hexagonally structured cell culture substrate. When the cells are descending onto the substrate surface, they would slip into and be trapped by the grooves between solid spheres to form a hexagonal pattern.

B. Cell preparation

The cells used here are the mouse monocyte-macrophage cells (RAW cells), which are one kind of semi-adherent cells. We can take advantage of the suspension feature of RAW cells before adhesion to demonstrate the cell patterning of suspended cells. Furthermore, the adhesion feature can be used to topographically study the behavior of adhered RAW cells on our cell culture substrates. The RAW cells were cultured in Roswell Park Memorial Institute medium (RPMI, Invitrogen) at 37 °C and 5% CO₂ environment. The concentration of the cell was about 140×10^4 cells/ml. The dye exclusion test was conducted to verify the cell viability on the cell culture substrate. The cells were stained by Trypan blue (Invitrogen, 0.4% Trypan blue mixed with RPMI 1:1), which only infiltrated the dead cells and stained them blue, leaving the living cells unstained, and thereby allowing observation of the cell viability.

III. RESULTS AND DISCUSSION

Before commencing the cell patterning, we first discuss the topographic features of the cell culture substrate. As mentioned earlier, our fabrication method has the benefit that we can create cell culture substrates of different unit patterning areas just by adjusting the volumetric flow rate of the disperse phase fluid and continuous phase fluid, which determine the size of the

FIG. 2. (a) and (b) Schematic of surface modification by PDMS for biocompatibility. (c) The schematic of cell patterning on the hexagonally packed cell culture substrate.

TPGDA droplets composing the patterning areas. Figure 3(a) shows the variation of TPGDA droplet's diameter with Q when $Q_w = 1.0 \mu\text{l/min}$, $1.5 \mu\text{l/min}$, and $2.0 \mu\text{l/min}$, where $Q = Q_T/Q_w$ means the flow rate ratio of the flow rate of TPGDA Q_T to the flow rate of water Q_w . We measured the droplet diameter from the pictures taken by an optical microscope. The diameters for multiple (at least 30) droplets were measured, and the averaged diameter and standard deviation were derived. The resolution of our diameter measurement was about $0.6 \mu\text{m}$. The measurement of the droplet's diameter was carried out after the droplets entered the expanding section (see Fig. 1). As Fig. 3(a) shows, for a constant Q_w , the droplet diameter and its standard deviation varied proportionally to the value of Q ,³⁷ and the deviation became significantly large beyond the corresponding Q s marked by the dashed lines for each Q_w . For example, when $Q_w = 1.0 \mu\text{l/min}$ and Q was smaller than or equal to 1.4, the droplet diameter and its deviation grew steadily from $77 \mu\text{m}$ to $98 \mu\text{m}$ and from $\pm 1.8 \mu\text{m}$ to $\pm 4.6 \mu\text{m}$, respectively. However, when Q reached 1.6, the droplet diameter increased dramatically to about $120 \pm 17.6 \mu\text{m}$. The notable deviations revealed the instability of the droplet generation, and in this situation, the droplet size was difficult to control.

FIG. 3. (a) Variation of TPGDA droplet's diameter with Q when $Q_w = 1.0 \mu\text{l/min}$, $1.5 \mu\text{l/min}$, and $2.0 \mu\text{l/min}$. The generation of droplets for each Q_w is unstable beyond its corresponding Q s marked by the dashed lines. (b-i) Schematic of the region for taking unstable droplet formation pictures. (b-ii) Sequential images of unstable droplet formation for $Q_w = 1.0 \mu\text{l/min}$ and $Q = 1.6$. For observation, the water is dyed with blue edible pigment.

In a T-junction microchannel, the transition from droplet squeezing to parallel flow streams is generally considered to be the threshold of unstable droplet formation. It has been shown that for a given capillary number Ca (defined as $Ca = \mu_w Q_w / \gamma A_w$, where μ_w is the viscosity of water, γ is the interfacial tension between water and TPGDA, and A_w is the cross area of the main channel), the unstable droplet formation can be triggered by increasing the flow rate of the dispersed fluid.^{38,39} On the other hand, for a smaller capillary number, a larger flow rate ratio of the dispersed phase to the continuous phase is needed to trigger the unstable droplet formation. Both of the above two trends can be found in our results shown in Fig. 3(a). Figure 3(b) shows the sequential images of the droplet formation at $Q_w = 1.0 \mu\text{l/min}$ and $Q = 1.6$. The dashed rectangle in Fig. 3(b-i) indicates the region where we took images. Figure 3(b-ii) shows that the TPGDA dispersed into multiple droplets after being dragged along the main channel for a long distance, and the formed droplets differed in size as shown in the image at 50.7 ms in Fig. 3(b-ii).

From the application point of view, the unstable droplet formation possesses no applied potential. Therefore, only the stable results will be discussed. From Fig. 3(a), we conclude the stable droplet formation occurred when $Q \leq 1.4$, 1.0, and 0.8 for $Q_w = 1.0$, 1.5, and 2.0 $\mu\text{l/min}$, respectively, it implies besides Q the Q_w also affected the size of the droplet. A larger Q_w led to the formation of smaller droplets at a constant Q when the droplet formation was stable. This means the size of the droplets is reciprocal to the capillary number Ca , which agrees with the results in previous literature.^{37,40,41}

Figure 4 shows the generation rate of the TPGDA droplets under different Q_w and Q when the droplets were stably generated. This figure shows that for a constant Q_w , the generation rate

FIG. 4. (a) Generation rate of the TPGDA droplets under different Q_w and Q when the droplets were stably generated. (b) Standard deviations of the droplet generation rate.

is proportional to the flow rate of the TPGDA Q_T , conversely, when Q is a constant, a larger capillary number result in a higher generation rate. The fastest and the slowest generation rates we recorded were 25 and 5 droplets per second, respectively, and the corresponding droplet diameter was $79.5 \pm 2.4 \mu\text{m}$ and $74.4 \pm 0.6 \mu\text{m}$, respectively. At generation rate of 25 droplets per second, it takes 17.3 h to cover a 10 cm diameter culture dish when only a single channel is used. However, the time can be significantly shortened to several minutes by using multiple channels, such as using 256 junctions as proposed in Ref. 42. Figure 4(b) shows the standard deviation of the droplet generation rate. Each of the standard deviation was derived from ten experiments. Comparing the standard deviations for different Q_w , it was observed that the standard deviation grew with the increasing of the capillary number Ca . Furthermore, for a given Ca , the standard deviation also increased when the flow rate ratio Q of the dispersed fluid increased. Note that the largest error bar of the droplet generation rate in Fig. 4(b) was only 0.81/s, which was 3.2% of the generation rate at that point. This means that the droplet generation rate could be controlled well when the droplet formation was stable.

We have to pay attention to another important factor that affects the cell culture substrate geometry, that is, the uniformity of the droplet size. Because the height of the microchannel was about $90 \mu\text{m}$, droplets larger than the channel height were deformed into non-spherical forms, making the groove depth between the droplets compressed. Therefore, we only focused on the cases where the droplet diameter was between $60 \mu\text{m}$ and $90 \mu\text{m}$. Figures 5(a)–5(c) represent the cell culture substrates assembled by TPGDA droplets generated under the conditions of (a) $Q_w = 2.0 \mu\text{l/min}$, $Q = 0.2$, (b) $Q_w = 1.5 \mu\text{l/min}$, $Q = 0.6$ and (c) $Q_w = 1.0 \mu\text{l/min}$, $Q = 0.8$, respectively. The average droplet diameters in Figs. 5(a)–5(c) were $60.5 \mu\text{m}$, $74.1 \mu\text{m}$, and $83.1 \mu\text{m}$, respectively. Figures 5(d)–5(f) show the distributions of droplet numbers over droplet diameters for the arrays in Figs. 5(a)–5(c), respectively. The droplet number of each bar in Figs. 5(d)–5(f) was summed over a range of $1 \mu\text{m}$ diameter distribution. The deviation of

FIG. 5. (a)–(c) TPGDA droplet array with droplet diameters of $60.5 \pm 1.5 \mu\text{m}$, $74.1 \pm 1.9 \mu\text{m}$, and $83.1 \pm 3.8 \mu\text{m}$, respectively. The scale bar indicates $100 \mu\text{m}$. (d)–(f) Distribution of droplet numbers over droplet diameters for the arrays in (a)–(c), respectively.

droplet diameter was only $\pm 2.5\%$, $\pm 3.8\%$, and $\pm 4.5\%$ for the droplet arrays in Figs. 5(a)–5(c), respectively, showing the high uniformity of the cell culture substrates.

Figure 6(a) shows a macro snapshot ($2 \times 2 \text{ mm}^2$) of a cell culture substrate composed of TPGDA spheres with diameter $83.1 \mu\text{m} \pm 3.8 \mu\text{m}$, which were in a highly ordered hexagonally packed structure. However, for a large area fabrication, there might appear some defects, voids and dislocations as shown in Fig. 6(b). The defects of the TPGDA sphere array were caused by merged droplets, and one or two defects led to a dislocation in the sphere array. On average, the percentage of defects and voids of a sphere array with $2 \times 2 \text{ mm}^2$ area was between 0.09% and 0.38%.

To obtain the desired pattern of cells in our passive cell patterning, the grooves between TPGDA spheres are required to possess a certain degree of depth, so it is important to discuss the depth of the grooves. In our experiment, the grooves might be filled with PDMS during the surface modification, and this resulted in function failure of the cell patterning of the substrate. Therefore, we diluted the PDMS to make the PDMS covered only the surface of the TPGDA spheres instead of filling the grooves. Figure 7(a) shows the PDMS-modified substrate surface, and the definition of the depth s of the grooves for cell patterning is shown in Fig. 7(b). To measure the groove depth, we first deposited a thin Cu metal layer (about 100 nm) on the PDMS layer for reflection purpose. Then we used a metallographic microscope to probe the focal planes of the sphere top and the groove bottom, and the groove depth s was derived by subtracting the distances of the two focal planes. The resolution of the groove depth measurement was about $1 \mu\text{m}$, which was determined by the fine-tune precision of the focusing function of our metallographic microscope.

Figure 7(c) shows the measured groove depth of cell culture substrates composed of different sized TPGDA spheres. Each data point in Fig. 7(c) was the average value derived from over 20 measurements on a substrate. The smallest s we measured was $13.9 \pm 1 \mu\text{m}$ when the average diameter of the TPGDA droplets was $60.5 \mu\text{m}$, and it was still large enough to trap suspended RAW cells that are $10\text{--}20 \mu\text{m}$ in diameter. Furthermore, it can be observed from the error bars that the variation of s was smaller than $3 \mu\text{m}$ for each substrate, which again indicates the high uniformity of our cell culture substrates. In addition, we also measured the thickness of PDMS coating under an optical microscope, and the thickness was measured as $2.2 \pm 0.5 \mu\text{m}$ for all substrates. We found the thickness of PDMS layer was only dependent on the air-dry time before baking, and the $2.2 \pm 0.5 \mu\text{m}$ PDMS thickness was the result of 5 min air-dry time. Although the variation of PDMS thickness was as large as 45.5%, but the PDMS thickness was too small to impact the surface profile of the cell culture substrate. This means the surface profile of our cell culture substrate is highly reproducible.

Figure 8 shows the cell patterning results. The average diameter of the TPGDA spheres was $83.1 \mu\text{m}$. Figure 8(a) shows the pattern of cells that have just descended onto the surface and been slightly washed. Because the suspension of the RAW cells, they could slip into the patterned grooves by gravity, and it is obvious that the remaining cells formed a hexagonal

FIG. 6. (a) $2 \times 2 \text{ mm}^2$ snapshot of a cell culture substrate composed of TPGDA spheres with diameter $83.1 \pm 3.8 \mu\text{m}$. (b) Defect, void, and dislocation of a large fabricated cell culture substrate.

FIG. 7. (a) The PDMS-modified surface of the cell culture substrate. The scale bar indicates 60 μm . (b) Definition of the depth s of the grooves for cell patterning. (c) Variation of groove depth with TPDGA sphere size.

pattern. Figure 8(b) shows the result after cells had been cultured on the substrate for 24 h, and Fig. 8(c) is the macro shot of Fig. 8(b). As Fig. 8(c) shows testing by Trypan blue staining demonstrated nearly all the cells to have survived; only a few dead cells (marked by dashed circles) were present that exhibited dark blue staining. There were 191 living cells and 12 dead cells in Fig. 8(c) and the cell viability was 94.1%. In addition, the cells tended to grow on two sides of the grooves in Figs. 8(b) and 8(c) instead of right on the groove lines. To study the cell distribution more quantitatively, we defined a cell patterning rate as a way of analyzing the cell distribution. Definition of the cell patterning rate was illustrated in Fig. 8(d). The left-hand-side plot of Fig. 8(d) shows the cell patterning region, which included the regions extending from the groove line and the triangular space between three adjacent spheres. The distance between the extended region boundary and the groove line was set to be the same as the average diameter 15 μm of RAW cells. Based on the cell patterning region, the cell patterning rate can be defined as $N_{\text{in}}/N_{\text{total}}$, where N_{in} was the number of cells inside the cell patterning region, and N_{total} was the total number of cells. The estimated cell patterning rate was 100% in Fig. 8(a), and was about 94.8% in Fig. 8(b) and 92.5% in Fig. 8(c). The overall cell patterning rate was estimated to be about 91.2% after cells had been cultured on the substrate for 24 h.

For future application of the RAW cell pattern on our cell culture substrate, it is significant that antigen presenting RAW cells can bind with matching helper T-cells, facilitating the use of the patterned antigen presenting RAW cells to construct patterns of T-cells for immunoassay. In addition, when the cells are suffering flush, as for example, in a microchannel with flowing fluid, the cells can hide or be stuck within the patterned grooves on the cell culture substrate.

FIG. 8. (a) The cell patterning result of just after the cells descending onto the surface. (b) and (c) The cell patterning result after cells had been cultured on the substrate for 24 h. The scale bar indicates $50\text{ }\mu\text{m}$. The dashed circles in (c) mark the dead cells, and the other cells are living cells. (d) Schematic of the definition of cell patterning region.

This benefits the maintenance of the cell pattern and those unbonded cells are washed away. Furthermore, when RAW cells keep growing till confluent, the cells may spread all over the TPGDA hill surface to form another kind of pattern. This kind of cell pattern could be applied to mimic the villi structure of small intestine.

IV. CONCLUSIONS

We demonstrated the fabrication of ordered cell culture substrates by a T-junction microfluidic channel. Our method possesses a promising advantage of actively tuning substrate dimensions for rapid experimentation. In addition, the results of the substrate dimension analysis verify that the cell culture substrates were fabricated with very high spatial uniformity. The cell patterning function of our cell culture substrates has also been proved by the patterning results of RAW cells. It shows that the RAW cells could be well patterned in hexagonal patterns when cells were suspended. The high viability and patterning rate of proliferated cells also indicate the feasibility of our cell culture substrates.

For the future application of our proposed method, hexagonal contact guidance provided by our fabricated cell culture substrates may be useful for some topographical biology research on cells like fibroblasts, neuroblasts, epithelial cells, or muscle cells. Furthermore, there is a tremendous opportunity for fabricating the cell culture substrates with biomaterials that can be chemically modified to promote cell growth. For example, one can produce PDMS droplet arrays as masters for molding cell culture substrates of biomaterials like collagen or matrigel.

One can also use polyethylene glycol diacrylate (PEGDA), which is also a UV sensitive material, as the dispersed phase to produce a topographically interesting substrate that prevents cell attachment. Our approach may provide useful information for future studies on cell biology.

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