Microfluidic Micropillar Arrays for 3D Cell Culture

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Abstract: Cell-based assays are one of the most important steps to select huge amount of drug candidates in drug discovery. To get more credible assay results, cell culture in the form of microscale environment and three-dimension has been exploited by microfluidic hydrodynamic focusing. However, the method still needs an enhanced reliability of scaffold formation and fast cell immobilization in a microchannel. In this report, we fabricated a microfluidic micropillar arrays (MMA) platform for cell culture using a poly(dimethylsiloxane) (PDMS) replica molding process. Peptide hydrogel and Matrigel were nicely patterned along the micropillars by surface tension. In addition, a linear concentration gradient profile was presented in a stripe-shaped Matrigel matrix and the simulation result with computational fluid dynamics (CFD) solver was corresponded to the experimental profile. The MMA platform was successfully applied to the hepatocellular carcinoma cell (HepG2) culture for 2 days.

Key Words: 3D cell culture, Micropillar arrays, Microfluidics, Hydrogel, Concentration gradient.

INTRODUCTION

Recently, as microelectromechanical systems (MEMS) and microfluidics technologies have been remarkably developed, various cellular microsystems were reported to support *in vivo*-like microenvironment and more precise cell manipulation [1-7]. Since microscale systems are able to control extracellular microenvironment that plays a significant role in cell signaling, cell to cell interaction and transportation of molecules [8,9], microfluidic platforms to create three-dimensional (3D) microenvironment have been widely studied using various kinds of hydrogel [10-14]. In addition, microfluidic tools are able to contribute a sophisticate and precise construction for cell matrix as well as an automatic operation and manipulation of cells, so that realization of more credible cell-based assays and high throughput screening is one of the critical advantages [15-20]. Therefore, many efforts have been progressed to conduct microscale cell-based assays in state of 3D cell culture.

Toh *et al.* developed a microfluidic perfusion system supporting mammalian cells with an adequate 3D cell–cell and cell–matrix interaction by using a micropillar array [13]. They immobilized cells through the use of pump withdrawal while the center outlet kept closed. However, cells were sometimes damaged during the cell immobilization depending on the flow rate. In our previous study, we developed a microfluidic device for 3D cell culture as well as *in situ* cell-based assays by hydrodynamic focusing [10,21]. However, it did not guarantee the reliability of scaffold width which was critical in the formation of concentration gradient. In addition, relatively big shear stress and exposure of distilled water to cells were regarded as harmful factors.

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Here, we demonstrate a microfluidic micropillar arrays (MMA) platform to realize stable 3D cell immobilization and *in situ* cell-based assays by generating a linear concentration gradient profile. Surface tension was used to pattern hydrogels, so that micropillar arrays were built in the middle of the main channel of a MMA device. In this report, a novel way to fabricate a stripe-shaped hydrogel matrix is introduced. A concentration gradient profile in a hydrogel matrix, computational fluid dynamics (CFD) simulation for the profile and hepatocellular carcinoma cell (HepG2) culture in a microchannel will also be discussed.

MATERIALS AND METHODS

Principle and Procedure for Forming a Stripe-Shaped Hydrogel Matrix

To fabricate a stripe-shaped hydrogel matrix, gas–liquid two phase surface tension was used. Surface tension is the phenomenon that liquid molecules at the surface are attracted inward and to the side, no outward attraction to minimize free energy of the system. When radii of curvature of the surface along any two orthogonal tangents are similar, the balance of the tension forces with pressure is expressed by the Young–Laplace equation:

$$\Delta p = \frac{2\sigma}{r} \quad (1)$$

where Δp, σ, r represent the pressure difference, the surface tension and the radius of curvature in each of the axes that are parallel to the surface, respectively.

As shown in Fig. (**1a**), micropillar arrays were straightly built in the middle of the main channel and the mixture of a hydrogel and cells, media and drugs are connected to the inlet ports. Then, the mixture is slowly flowed *via* the center

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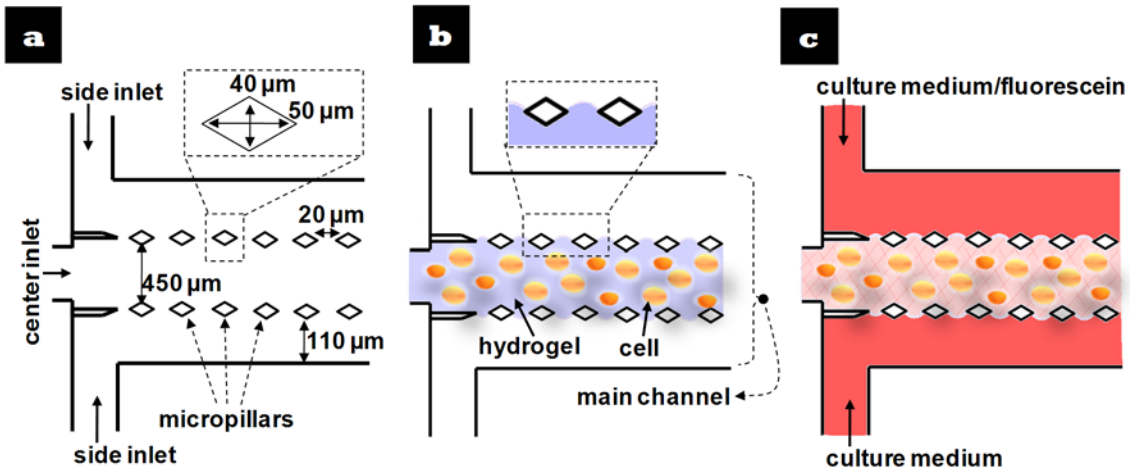

Fig. (1). Schematic procedure for fabricating a stripe-shaped hydrogel matrix. (**a**) A microfluidic device having diamond-shaped micropillar arrays in the middle of the main channel of a MMA device (**b**) Hydrogel containing cells is flowed *via* center inlet of the main channel. Any kinds of hydrogels can be patterned. (**c**) Culture medium or drug solution is flowed *via* both sides of inlets.

inlet port (Fig. **1b**). If the conditions of the gap between micropillars (r) and the flow rate of mixture (Δp) are satisfied to sustain surface tension of the mixture, the flow is proceeded to parallel direction. When the fluid is reached to the outlet, media are flowed *via* both side inlet ports (Fig. **1c**). At this time, the media are diffused to the hydrogel–cell mixture, so that nutrients required for cell culture are supplied to the cells.

For fabrication of a stripe-shaped matrix, 1% (w/v) Puramatrix (BD Biosciences, MA) that is a sol-gel transition peptide hydrogel, and Matrigel (BD Biosciences) that is a thermosensitive hydrogel, were applied to verify availability of different kinds of hydrogels. When Puramatrix is contacted with electrolyte solution, it is transitioned from sol to gel. Therefore, once media were flowed *via* both side channels to contact the Puramatrix mixture, the hydrogel was simply gelled. More details for the gelation of Puramatrix are referred to the previous works [10,21]. On the other hand, since Matrigel is sensitive to temperature, it should be maintained at below 4 °C, even in a syringe tube. When the Matrigel was inserted to the main channel of a MMA device, the temperature was raised up to 37 °C as quickly as possible to make it be gelled.

Microfabrication of a MMA Device

A MMA device was fabricated by photolithography and a poly(dimethylsiloxane) (PDMS) replica molding process (Fig. **2**). The height of microchannels was 50 μm to satisfy 3D microenvironment and easy optical observation. The gap between micropillars was designed with 20 μm and the length of micropillars was normally 50 μm (Fig. **1a**). A negative photoresist (PR), SU-8 2025 (MicroChem Corp., MA) was used to make micropatterns on a Si wafer. After photolithography process, a mixture of PDMS prepolymer and curing agent (Sylgard 184; Dow Corning, MA) was poured on the fabricated Si wafer and cured on a hot plate at 80 °C for 1 h. The PDMS replica was punched for the inlet and outlet ports and bonded with a slide glass by air plasma (200 mTorr, 200 W). Then, it was autoclaved and stored in an aseptic clean bench.

3D Cell Culture in a MMA Device

The hepatocellular carcinoma cell line (HepG2; ATCC HB8065) was chosen for 3D immobilization. HepG2 cells were maintained in a T75 (Falcon) flask inside a humidified water-jacketed incubator at 37 °C and 5% CO_2. Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS; Gibco) was used for HepG2 nutrient. After harvesting cells from the flask at 70%-80% confluence, the concentration of cell was set to 5×10^6 cells/ml. Then, Matrigel, regarded as a good scaffold, including abundant extracellular matrix proteins such as laminin, collagen IV and a variety of growth factors, was prepared within ice. The cooled Matrigel was mixed with harvested HepG2 cells and the mixture was transferred to cooled syringe. The mixture in the syringe was injected into a microchannel as quickly as possible, and then surrounding temperature was raised up to 37°C and media were flowed via the side inlets for 2 days. To examine cell viability check, calcein-AM (Invitrogen, OR) was flowed *via* the side inlet ports where viable cells represent green fluorescence.

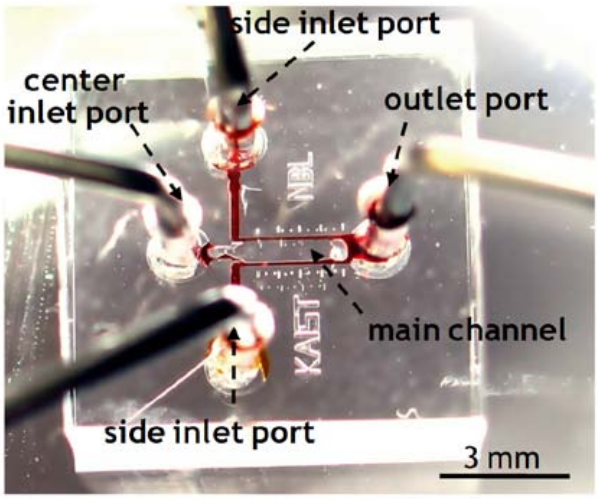

Fig. (2). A fabricated microfluidic micropillar arrays (MMA) device.

Analysis of a Concentration Gradient Profile within a Matrigel Matrix

After fabricating a stripe-shaped Matrigel matrix, green fluorescent fluorescein was flowed *via* one side inlet, and distilled water was flowed *via* another side inlet port. Pictures were taken at the interval of micropillars and analyzed by ImageJ program (W. Rasband, ImageJ 1.29× freeware, http://rsb.info.nih.gov/ij/). Acquired pictures were converted to 8-bit grayscale and the fluorescence intensity profile was plotted by the manual of "surface plot".

RESULTS AND DISCUSSION

Hydrogel Immobilization using Surface Tension

In the fabrication process of a MMA device, the design of micropillars was the major concern. Because the purpose of micropillars located in the middle of the main channel is to guide the flow of mixture, they should be as small as possible. However, the size of micropillars should be considered and optimized with the channel height of the MMA device. In this study, the micropillar size was over at least 20 μm unless the photoresist for micropillars was not completely developed. Various shapes of micropillars were made including triangle, square, ellipse and diamond shapes. Though all of micropillars made hydrogel to be patterned, diamond shape of micropillars was more proper for no generation of bubbles.

In addition, since the gap between micropillars was related to the radius of curvature of mixture surface in Young–Laplace equation, it was also critical factor for successful micropatterning of a hydrogel. Therefore, it was set to 20 μm distance, so that micropatterning could be succeeded even in relatively fast mixture injection (relatively big pressure difference). This advantage is particularly important when using a peptide hydrogel. Since the pH of peptide hydrogel is around 3, the condition is harmful to cells. Therefore, peptide hydrogel should be injected as quickly as possible [10]. To satisfy the step, the MMA device was designed to operate even in rough manual injection with a syringe.

Fig. (**3**) shows a typical process of micro patterning for the peptide hydrogel. In a bare main channel of the MMA device (Fig. **3a**), the mixture of microbeads and Puramatrix was manually injected. As shown in Fig. (**3b**), the mixture was flowed along the micropillars. When the mixture was reached to the outlet port of the main channel (Fig. **3c**), both sides of media were flowed (Figs. **3d** and **3e**). Since Puramatrix is gelled when contacted with electrolyte solution, thread-like patterns could be observed (Fig. **3f**). Similar to Puramatrix procedure, we could also fabricate a stripe-shaped Matrigel matrix (data not shown). Instead, after flowing Matrigel *via* center inlet port, it should be waited until gelation with heating (37 °C) in that Matrigel is a kind of a thermosensitive hydrogel. Compared to our previous study [10] which performed the construction of a stripe-shaped peptide hydrogel by hydrodynamic focusing, the MMA device simply realized the fabrication of microscale scaffold. In addition, the width of hydrogel scaffold was very reliable. Moreover, since this approach does not require distilled water to control the gelation of Puramatrix, it can be regarded as a more biocompatible method for cells.

Concentration Gradient Profile in a Micropillar-Guided Channel

After forming the Matrigel matrix in a MMA device, a concentration gradient profile was analyzed. The flow rate of both sheath fluids was around 30 μl/h and the interval area of micropillars was observed. Actually, we speculated that a linear concentration profile might not be shown in the width of a Matrigel matrix because micropillars existed in the main channel can make dissipate stream lines of sheath fluids. However, as shown in Figs. (**4a** and **4b**), a linear concentra-

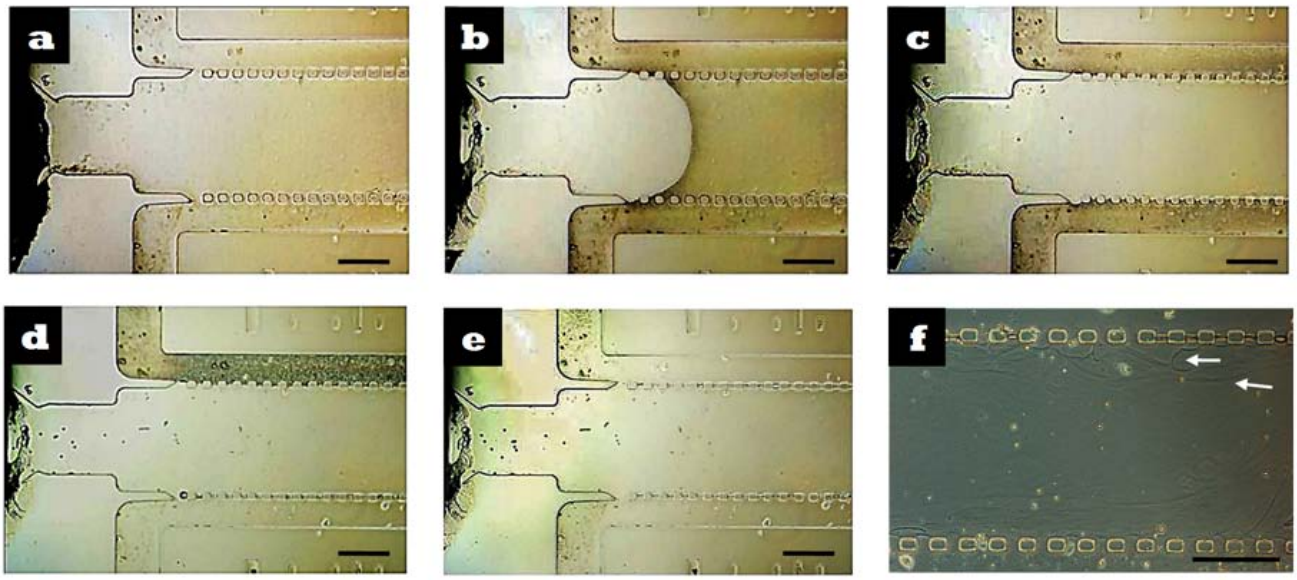

Fig. (3). Micropatterning of peptide hydrogel using surface tension. (**a**) A bare microchannel having square-shaped micropillar arrays. (**b**) Mixture of Puramatrix and 7 μm polystyrene beads (Polysciences Inc., PA) was manually injected in the middle of inlet port by a syringe. (**c**) The mixture was flowed along to the guide of micropillar arrays, reached to outlet port. (**d**) After confirming hydrogel patterning, medium was flowed *via* the downward inlet port. (**e**) Medium was flowed *via* upper inlet port. (**f**) Puramatrix was instantly gelled by media and thread-like lines were shown as being gelation (see the white arrows). All scale bars are 200 μm.

tion gradient was formed in the Matrigel matrix. The linear profile was normally reached at steady-state within 10 s and continuously maintained if some fluctuations by syringe pumps are neglected. This tendency was corresponded with our previous work [22]. We studied a concentration gradient profile with commercial CFD solver (CFD-ACE; ESI, Huntsville, AL). The geometry and dimension used in simulation was almost the same with the fabricated MMA device. "Flow" and "User scalar" modules were used in CFD-ACE to examine a concentration profile.

Fig. (**4c**) shows a scalar distribution in the main channel of a MMA device where the red color represents fluorescent solution. Similar to the experimental result shown by the open circle line in Fig. (**4d**), a linear concentration gradient profile was represented at the interval of micropillars (the closed square line of Fig. **4d**). Actually, we also simulated a concentration profile with the microchannel design having square-shaped micropillars. However, it was not affected on concentration profile. One of major reasons for no difference of concentration profile seems that the flow rate of sheath fluids is relatively low, so that the flow rate in the matrix was almost zero. This implies that a concentration profile is governed by the Fick's first law which is dependent on only boundary concentration, not for design or geometry.

3D Cell Culture in a Matrigel Matrix with a MMA Device

To cultivate cells in a microchannel, maintaining aseptic state for a MMA device, syringes, tubes and outlet reservoirs was important. When using Matrigel as a matrix, it should be waited until being gelation. In the product specification sheet of Matrigel provided by the company, a 30 min waiting period was recommended for gelation. However, cell viability was very low when performed according to the recommendation. Because the amount of volume inserted into a microchannel was less than 100 pl, it was very easy to be evaporated. The phenomenon could be seen in the microchannel (see the changed black spots in the upper sheath channel area of Fig. **3d**). Thus, media were flowed *via* both sides of inlets after waiting for 1 min and the matrix was fairly formed. Actually, we doubted whether maintaining cells under 4 °C was contributed to worse cell viability or not. As a result, it was little affected on cell viability during 10 min exposure. Fig. (**5a**) shows HepG2 cells 3-dimensionally immobilized along the micropillars. The cells were cultivated on a humidified water-jacketed incubator at 37 °C and 5% CO_2. Since a syringe pump was located in the incubator, nutrients were supplied with perfusion. As shown in Figs. (**5b** and **5c**), cells were fairly proliferated and the viability was about 97%. The most critical problem to cultivate cells was whether any bubbles are created in a microchannel. Normally, high pressure-driven fluid injection to a microchannel is able to remove remnant micro bubbles [23]. In this case, however, any fluid injection was not permitted before flowing a hydrogel *via* the center inlet port because surface tension effect was disappeared. Therefore, micropillar shape was more critical in cell culture, so that a microchannel having diamond-shaped micropillars was the best option in 3D cell culture.

CONCLUSIONS

In this study, we developed a MMA platform by photolithography and the PDMS replica molding process. Peptide hydrogel and Matrigel were nicely patterned along the micropillars by surface tension. In addition, a linear concentration gradient profile was presented in a stripe-shaped Matrigel matrix and the simulation result with CFD solver was

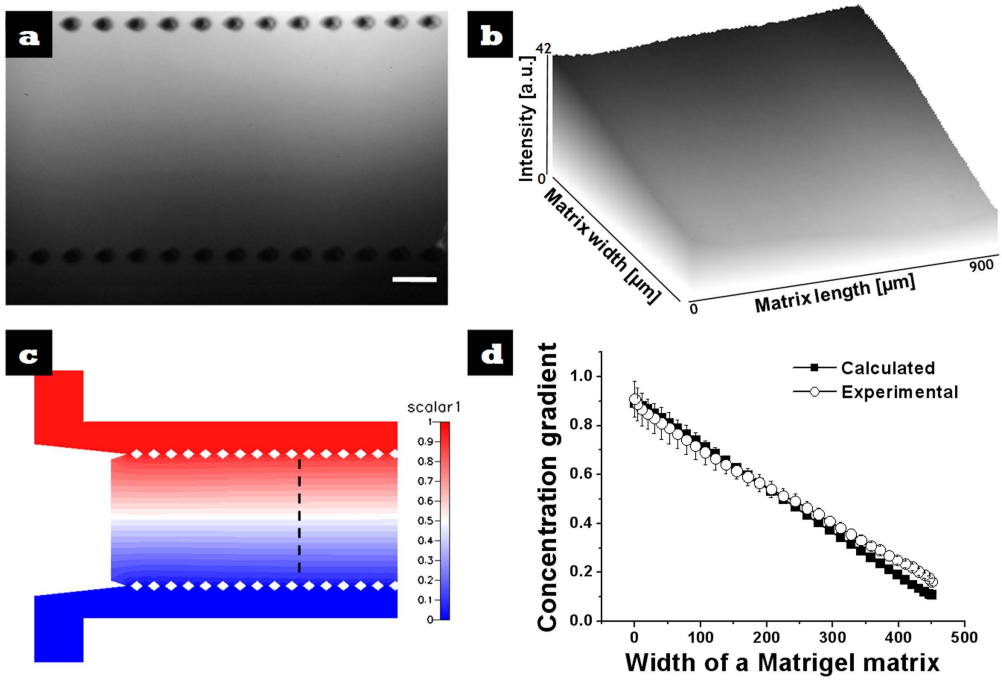

Fig. (4). Analysis of concentration gradient profile in a Matrigel matrix formed in a diamond-shaped MMA device. (**a**) A fluorescent picture forming concentration gradient in a Matrigel matrix. Scale bar is 100 μm. (**b**) Surface plotted graph of fluorescence intensity at the interval of micropillars. (**c**) Scalar distribution in the micropillar geometry by numerical simulation. (**d**) Plot of the experimental and calculated concentration profile about the vertical dotted line of the panel c.

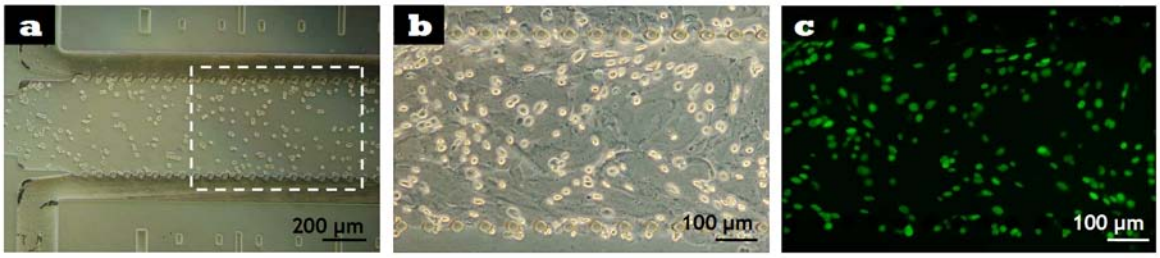

Fig. (5). Cell culture in a Matrigel matrix formed in a diamond-shaped MMA device. (**a**) A picture of 3D immobilized HepG2 cells in a Matrigel matrix. (**b**) The image shows HepG2 cell morphologies for the white square area of the panel a after 2 days. (**c**) The fluorescence image of calcein-AM in the panel b.

corresponded to the experimental profile. HepG2 cells were cultured for 2 days in a MMA device and most cells were viable. Therefore, it is expected that the MMA platform would be useful for various dose-dependent cell-based assays in state of 3D cell culture. In addition, since the MMA method is based on physical patterning, various kinds of hydrogel, including pH sensitive, thermosensitive and photosensitive hydrogels, can be patterned by the same micropillar geometry.

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