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Micromolding of PDMS scaffolds and microwells for tissue culture and cell patterning: A new method of microfabrication by the self-assembled micropatterns of diblock copolymer micelles

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Abstract

We introduce an innovative fabrication of the polymer scaffolds for tissue culture by utilizing the evaporation induced self-assembled micropatterns of polystyrene-*block*-poly(acrylic acid) (PS-*b*-PAA) diblock copolymer micelles. The microstructures were used as templates for micromolding a silicon elastomer, poly(dimethylsiloxane) (PDMS), into tissue scaffolds and microwells for cell patterning purpose. Cultivation of human epithelial cells (Calu-3 cell line) on the PDMS scaffolds demonstrates potential applications in tissue engineering and cell-based biosensors. The reported method is rapid, simple, economical, and versatile comparing with the existing microfabrication techniques.

Keywords: Micromolding; Microwells; PDMS scaffold

1. Introduction

In the up front miniaturizing technology, microfabrication has become an indispensable technique for a variety of scientific research and engineering development. The state-of-the-art microlithographic techniques facilitate advancements in various fields, such as microelectromechanical systems (MEMS), miniature electrical devices and sensors in microelectronics [1–3]; lab-on-a-chip and microarray-exploration in genomics and proteomics [4]; and biochemical assays and clinical diagnostics in medical science [5,6]. The significance of microfabrication attributes to the rapid and mass scale manufacture of micro devices and their components in microscopic scale. To perform the fabrication, however, a large-scale operation and sophisticated instrumentation are usually involved. Another disadvantage lies in its complicated processing procedure, such as thin-film deposition and chemical etching, in the conventional lithography. In this paper, we introduce a novel microfabrication technique by employing the evaporation induced self-assembly of diblock copolymer micelles into well-ordered microstructures. By using the obtained micropatterns as templates or moulds for micromolding micromeshes and microwells, biocompatible or biodegradable polymers can be fabricated by much simple procedures when compared with the conventional manufacturing approaches. The achieved microscopic structures can be applied as tissue culture scaffolds and substrates for cell patterning or cell-based biosensors. Polymeric microwells to be developed for bead-based analytics are also fabricated and will be discussed in the content.

The evaporation process of colloidal suspensions induces morphological instability and the remaining dried materials often show patterns of cracks. Examples can be found in everyday life such as cracks appeared in dried paint, latex, and mud. In the past decades, both mathematical and experimental studies have been carried out to investigate the cracking phenomenon [7–12]. In our experiment, a micelle solution of polystyrene-*block*-poly(acrylic acid) (PS-*b*-PAA) diblock copolymer, which shows a distinct dry-cracking phenomenon, was used for the crack pattern formation [13–15]. The crack patterns, which were in micron scale, were then used as templates for microfabricating various polymeric structures. The objective of this study is to make use of the dried micelles micropatterns as moulds for micromolding polymeric

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microwells for tissue culture scaffolds, cell patterning platforms, and also bioanalytics devices.

2. Material and method

2.1. Formation of diblock copolymer micelles

Polystyrene-*block*-poly(acrylic acid) (PS-*b*-PAA) diblock copolymer (P1541-SAA) with M_n: PS(66500) and PAA(4500) and M_w/M_n: 1.08, was purchased from Polymer Source, Inc., Quebec, Canada. PS-*b*-PAA was dissolved in a common solvent, tetrahydrofuran (THF) to a concentration of 10 mg/ml. Micellation was performed by dropwise addition of the selective solvent, deionised water (NanoPure Diamond Ultrapure Water System), at a rate of 1 drop every 10 s under slow stirring until a final concentration of 0.09 v/v was reached. The morphology and the diameter of micelles, which plays a determining role of the micropatterns formation, were studied by Transmission Electron Microscopy (TEM) (Jeol 100CXII) and Scanning Electron Microscopy (SEM) (Jeol 6300F).

2.2. Fabrication of diblock copolymer micropatterns

N-Type silicon wafers were cut into pieces of 5 mm × 5 mm, and were used as substrates for the self-assembly of dried micelles into micropatterns. The substrates were rinsed by isopropanol, followed by copious amount of nanopure water and were blow dried by compressed nitrogen gas. A drop of 15 μl micelle solution was dropped onto a single substrate and was allowed to evaporate at ambient condition. Formation of micropatterns was confirmed by Scanning Electron Microscopy and optical microscopy (Olympus BX-41, Japan, equipped with a Leica DC100 CCD camera).

2.3. Micromolding of PDMS microwells

Low viscosity poly(dimethylsiloxane) (PDMS) (Elastosil 601, Wacker AG, Germany) was mixed in 10:1 weight ratio (silicone rubber: curing agent). A drop of 0.5 μl silicone rubber mixture was micropipetted onto the micropattern. A microscopic cover g lass, previously cleaned with isopropanol and nanopure water, was laid on top of the silicone rubber, leading to a homogeneous coverage of the low viscosity PDMS over the micropattern. The molding PDMS/micropattern sandwich was kept in a vacuum oven overnight for degassing and solidification. The PDMS/micropattern sandwich was then bathed in THF under slow shaking (MyLab Orbital Shaker SLOS20), resulting in the dissolution of the diblock copolymer micropattern and leaving the PDMS microwells adhered on the cover glass. The microwells were washed with nanopure water and were dried in vacuum oven to remove all the residue of organic solvent. The topology of the microwells was studied by SEM.

2.4. Cell culture on PDMS scaffolds/microwells

Human Calu-3 epithelial cells (HTT-55, American type culture collection (ATCC)), were grown in minimum essential medium MEM (Eagle) supplemented with 2 mM L-glutamine, and Earle's BSS containing 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, and 10% fetal bovine serum in an environment of 95% air and 5% CO_2 at 37 °C. Cells were trypsinized by 0.25% trypsin in EDTA once became confluence and were passaged by a subculture ratio of 1:3 every 3–5 days. For cell culture on PDMS scaffolds or microwells, the surfaces were sterilized by 70% ethanol followed by sonicating with nanopure water and then PBS buffer for 5 min. After cleaning, they were incubated in MEM for at least 24 h before performing the cell-seeding. Calu-3 cells suspended in culture medium at a concentration of 6×10^5 cell/ml were then seeded onto the scaffolds or the patterning surfaces.

3. Results and discussion

3.1. Micropatterns of polymer micelles

The PS-*b*-PAA diblock copolymer is amphiphilic in nature. During micellization, the selective solvent (water) will aggregate the hydrophilic PAA blocks and the micelles will be formed as illustrated in Fig. 1. As discussed earlier, the micelle solution will leave patterns upon drying. The patterns were studied by SEM (Fig. 2) and optical microscopy (OM) (Fig. 3), which shows the patterns are of micron scale. Different types of micropatterns were obtained with different morphologies, such as the orderly aligned cubic blocks (Figs. 2B and 3A), the rectangular blocks (Fig. 3B), the stripe-like structure (Fig. 3C), and the mesh-like structure (Fig. 3D). As revealed by SEM, micropatterns with distinct and explicit edges and right-angled corners are observed in Fig. 2B–D, and the cross-section of the patterns is shown in Fig. 2C. Surface profiler system (Alpha-Step 2000) was used for a more accurate measurement of the dimensions of and distances between the formed blocks. It was found that the width and height of the crack blocks were about 10 μm and the block-to-block distance (crack width) was about 1 μm. The TEM and SEM micrographs, c.f., Figs. 1b-ii and Fig. 2A, also reveal that only spherical micelles are capable in constituting regular micropatterns upon drying. The PS-*b*-PAA concentrations in THF for preparing the patterns shown in Fig. 3 were as following: 9.5–10 mg/ml for Fig. 3A, 9.2–9.5 mg/ml for Fig. 3B, 8.0–8.5 mg/ml for Fig. 3C. The pattern of Fig. 3D occurs nearly for all concentrations at out-skirt of the drying droplet when the drying wave is disturbed after longer propagation [13]. The cracking physics was discussed in our recent theoretical study on the formation mechanism and the control parameters of the drying induced crack patterns of the polymer micelle solutions [13].

3.2. Micromolding of the PDMS

Low viscosity curable poly(dimethylsiloxane) (PDMS) is a commonly used silicon elastomer in microfabrication [16–18]. It is highly flexible, chemically and thermally stable, and biocompatible. It is also well-known as an excellent soft

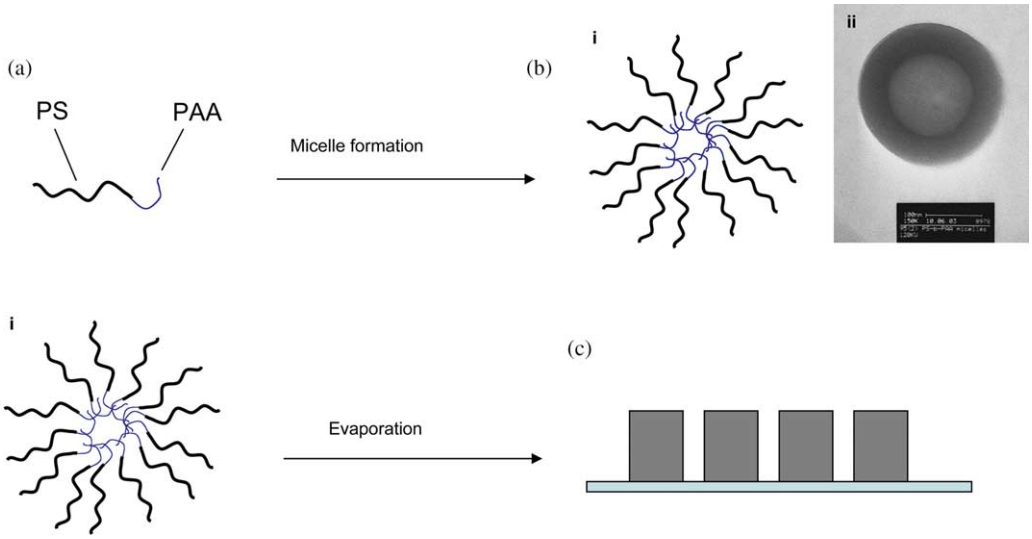

Fig. 1. (a) Polystyrene-*block*-poly(acrylic acid) diblock copolymer. (b-i) Self-assembly of diblock copolymer micelles. (b-ii) The TEM micrograph of a single micelle with a diameter of 30 nm. (c) The micropattern induced by evaporation.

material for soft-lithography [1,18–21], especially in biological and medical usage, due to a number of reasons: the high fidelity in moulding micron scale features; optical transparency; low curing temperature and fast curing; non-toxicity, enabling the cells to be cultured directly on the substrates; and also, the ease in controlling the surface chemistry by the conventional surface modification techniques for improved biocompatibility etc. [19]. In soft-lithograph, elastometric polymer, mostly PDMS, was cured in a master (or mould) previously fabricated by the traditional photolithography. The elastomer replicates the shape of the master with high fidelity and was peeled after curing. The obtained elastomeric structure with specific patterns on the surface (stamp) will then act as another pattern transferring agent. For most of the microfluidics and micromolding approach in soft-lithography, feature size from tens to hundreds micron is usually achievable. In our experiment, we adopt the merit of PDMS in processing elastomer microstructures, but with a much simpler and experimental friendly approach. Instead of performing the stepwise chemical treatment of photolithography in fabricating

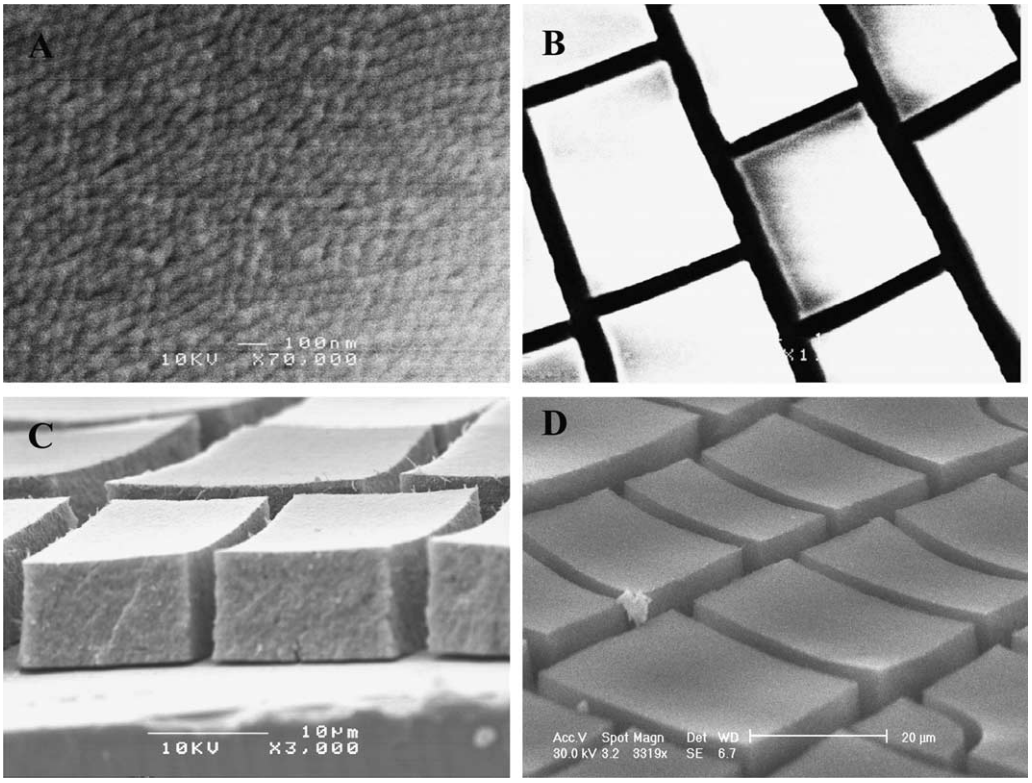

Fig. 2. (A) The SEM micrograph of aggregated micelles. (B, C, D) The SEM micrographs of diblock copolymer micropatterns induced by evaporation.

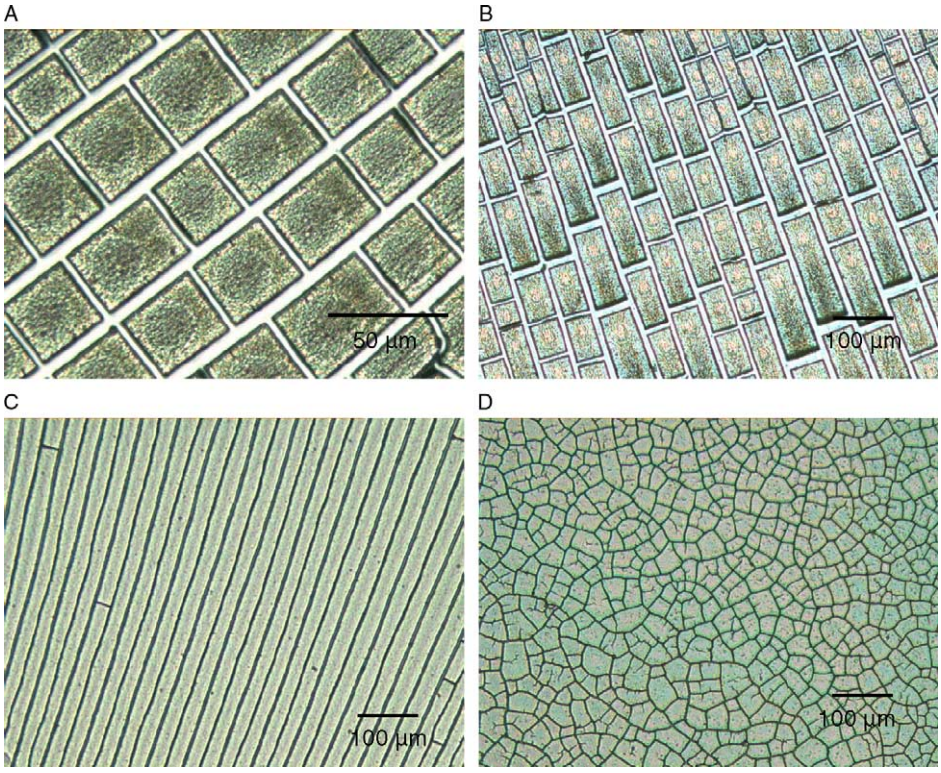

Fig. 3. The OM micrographs of micropatterns with square (A), rectangular (B), stripe-like (C) and mesh-like (D) structures, respectively.

the master for moulding, we can achieve similar templates or moulds by a one-step evaporation of diblock copolymer micelle solution. Micropatterns with comparable feature sizes (~10–50 μm) can be obtained in a few minutes. After that, a drop of mixture of PDMS base and curing agent (10:1) was cast onto the mould of micropatterns. A clean microscopic cover glass was laid on the top of the low viscosity PDMS as schematically illustrated in Fig. 4. The PDMS was cured under vacuum to remove the trapped air bubbles, and also, to enhance the flow of the elastomer, by both gravitational pull and capillary negative pressure, into the 1 μm-wide microchannels. After curing, the diblock copolymer mould was dissolved by THF, leaving the PDMS microwells adhered on the cover glass. SEM images and optical microscopic photographs of the well-like and mesh-like microstructures are shown in Figs. 5 and 6, respectively. The resulting microwells resemble the special features accomplished by the conventional micromolding or microfluidic techniques in the soft-lithography. They also inherit the advantageous physical, chemical, and structural properties for the application in biological and biochemical usage. In additions, no stringent processing environment, like clean-room facilities, sophisticated and expensive equipment, complicated procedures, or dangerous chemicals are needed. Polymeric microwells with the well dimension (length and width) of 10–50 μm, the depth of about 10 μm, and the wall of the well of about 1 μm were achieved, replicating the size of the micropatten templates with high fidelity. The removal of the mould, which is performed by the dissolution of diblock copolymer instead of the mechanical peeling, imposes no damage to the finished structures. The micromolding on diblock copolymer micropatterns can be applied to different thermal plastics for designated applications. For example, biodegradable cell culture scaffolds for tissue engineering [22–24] and also polymeric microwells as titer plates can be

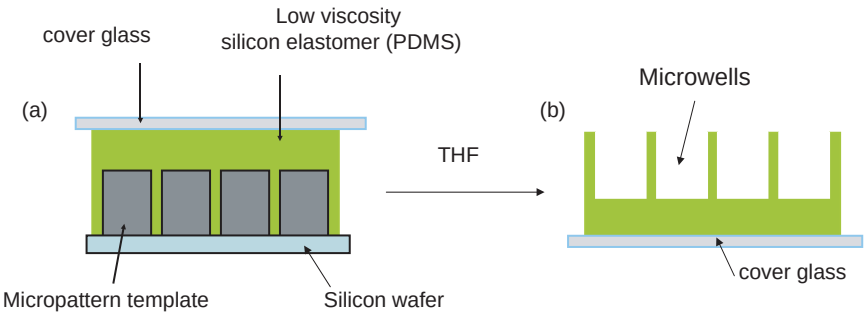

Fig. 4. Schematic diagram of the micromolding of PDMS microwells templated from diblock copolymer micropattern. (a) Micromolding of PDMS using diblock copolymer micropattern as a template. (b) The retained PDMS microwells after the removal of the diblock copolymer.

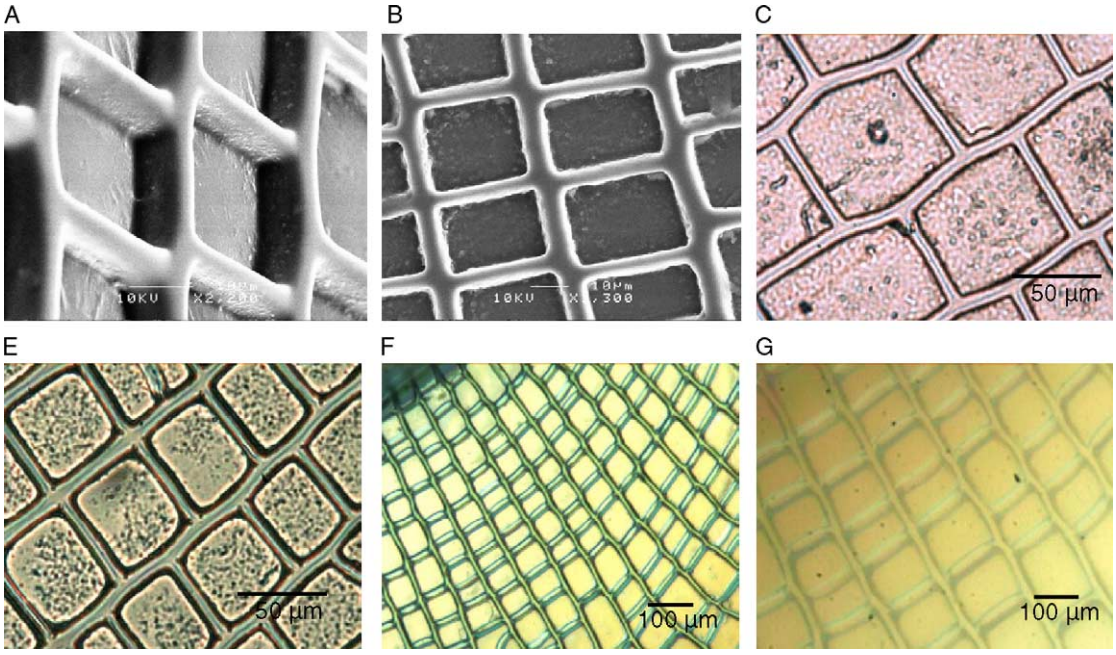

Fig. 5. (A, B) The SEM micrographs of the PDMS microwells. (C–F) The OM micrographs of the PDMS microwells.

fabricated to perform high-throughput assays for pharmaceutical and clinical analysis [5,6].

3.3. Microwells as cell culture scaffolds and substrates for cell patterning

One of the applications of this micromolding technique is the fabrication of polymeric scaffolds for tissue engineering [22,23]. A number of fabrication techniques, like the pressure-assisted microsyringe deposition [19] and the three-dimensional printing (3D^{PTM}) [22] for the poly(DL-lactide-*co*-glycolide) (PLGA) scaffold, the design and fabrication of polycaprolactone (PCL) scaffolds via Fused Deposition Modeling [25] and the state-of-the-art soft-lithography approach [4], have been reported in fabricating scaffolds for tissue engineering purposes. A number of methods have also been adopt in generating Most of the reported methods, however, encounter similar disadvantages as the modern microlithography does for the sophisticated facilities and the limited choice of the processing materials. Our micropattern templated micromolding technique offers an alternative to the conventional technology. In addition to the merit of fast, simple, and economical fabrication, the micromolded structures can also be processed with a wide range of materials. Both biocompatible and biodegradable polymers, either biosynthetic [26] or naturally derived [27], can be processed to satisfy specific condition of scaffolding. Such structures provide physiological friendly conditions for cell adhesion, migration and propagation by resembling the real biological environment. Human epithelial cells (Calu-3 cell-line) cultivated on the biocompatible silicone rubber microwells are shown in Figs. 7 and 8. The optical micrographs illustrate a favorable adhesion and healthy growth of the cells, as evidenced by the increase in the scaffold surface coverage by the cell colonies. One-cell-one-well adhesion was observed after 1-day culture as shown in Fig. 7. Fig. 8 shows an increasing area of cell coverage from the 1-day (Fig. 8A), the 5-day (Fig. 8B), the 7-day (Fig. 8C) to the 12-day (Fig. 8D–F).

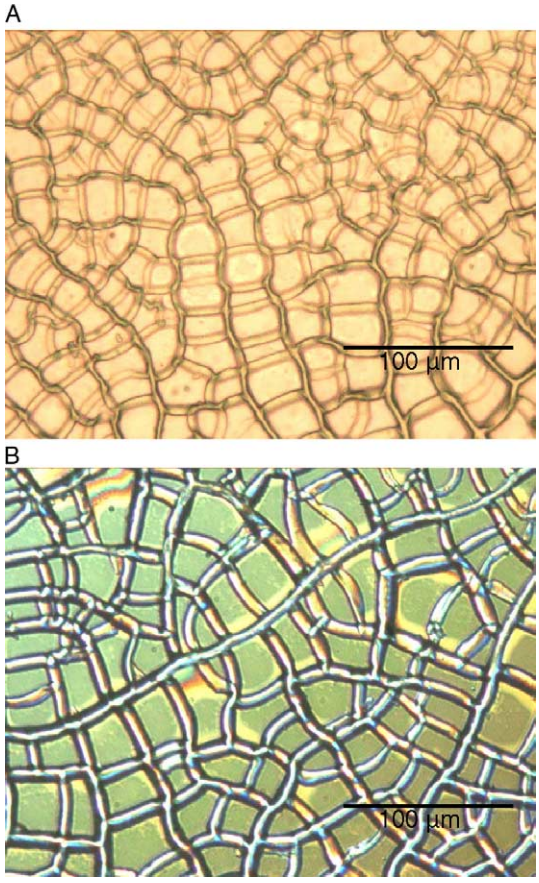

Fig. 6. The micromolded PDMS micromeshes.

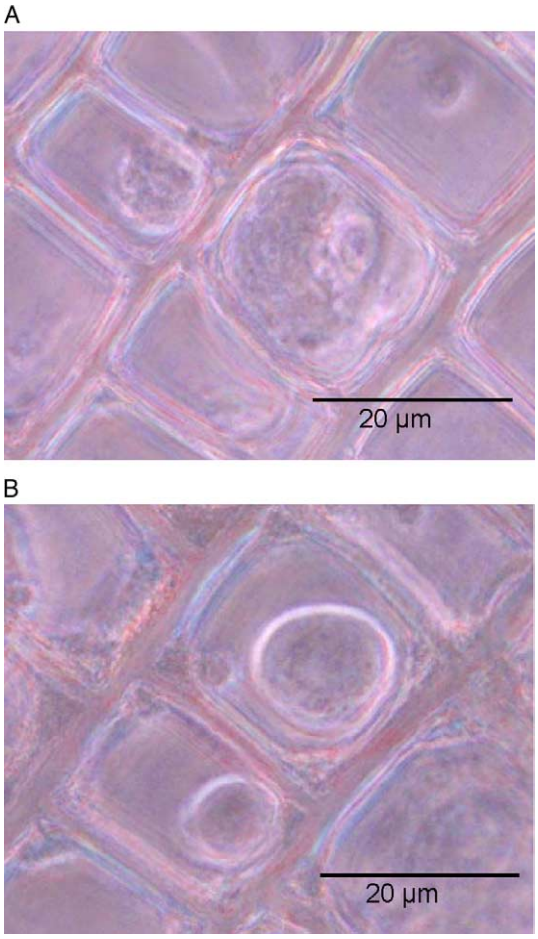

Fig. 7. Single-cell patterned in single microwell in the 1-day culture.

A layer of planar silicone rubber surface was prepared as a control substrate for culturing the same cell-line. As shown in Fig. 9, no cell adhesion was observed on the flat substrate after the 5-day culture, and no observation of further cell growth on the substrate until the cells were eventually died on the 12-day. These results suggest that the three-dimensional microstructure is essential for the adhesion and growth of the cells.

The micromolded PDMS microwells could be potentially utilized as the spatial confinements for patterning cells and proteins [28–32]. In the modern microfabrication, substrates with designated topology was mostly achieved by the conventional photolithography [4,28,31,32] or the more recently developed soft-lithography [29,30]. For this application, the diblock copolymer micropatterns tempated micromolding of PDMS microwells, offers a rapid and simple method in fabricating the patterning substrates. Our technique facilitates the microscopic study of cell–surface, cell–cell, and cell–medium interaction in potential [28–30]. Microwells containing single cells could be individually addressed and treated with medium, supplements, or agents to study single cell interaction and the cell growth and signalling. The study of cell proliferation and differentiation with various substrate topologies will be easier; for both the preparation time can be considerably reduced and processing procedures of the substrates under investigation can be simplified. In prospect, the technique could be developed as a valuable tool for miniaturized cellular arrays, cell-based biosensors, cell-patterning, and single-cell based research [33,34].

4. Conclusion

The novelty of this research is to utilize the drying induced micropatterns of diblock copolymer micelles as a convenient

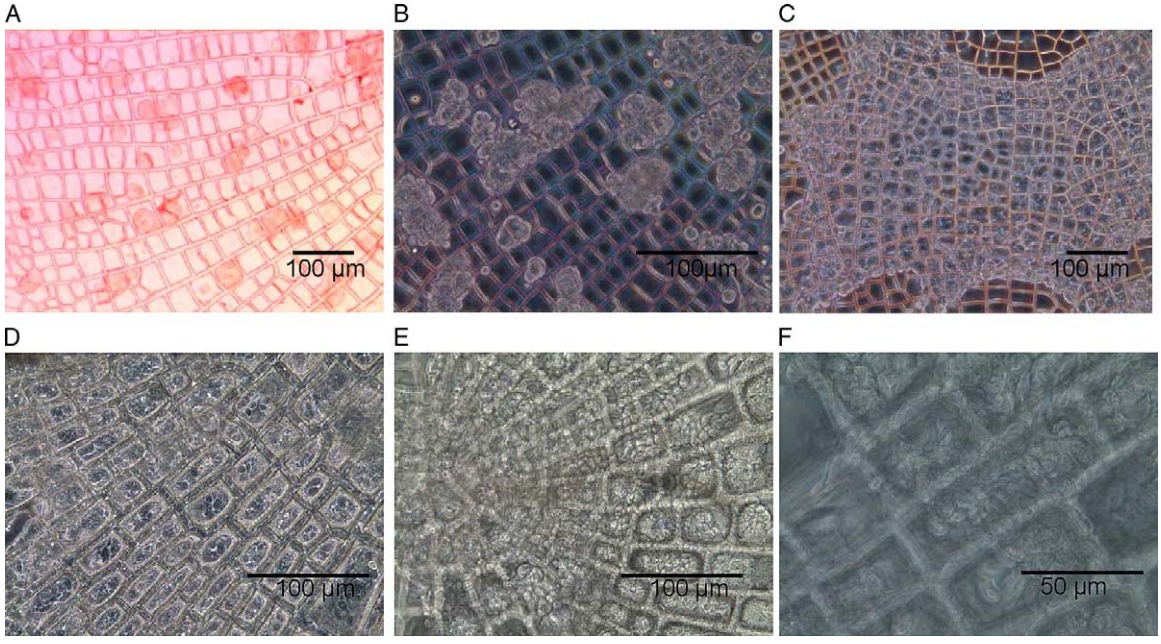

Fig. 8. Calu-3 cell-line culture on PDMS scaffolds showing an increasing surface coverage. (A) 1-day culture, (B) 5-day culture, (C) 7-day culture and (D–F) 12-day culture.

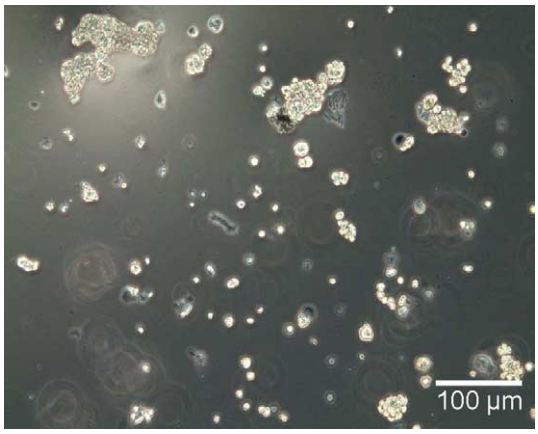

Fig. 9. The control experiment of the 5-day cell culture on a flat surface of the PDMS, which shows no cell adhesion. Cells suspended in culture medium were in intact spherical stage without spreading.

mould for micromoulding polymeric tissue culture scaffolds, cell patterning substrates, and platforms for bioanalytical microdevices. The model system we have applied is the micelle solution of polystyrene-*block*-poly(acrylic acid) (PS-*b*-PAA) diblock copolymer. PS-*b*-PAA micelle solution being dried on a substrate leading to the self-assembly of a number of orderly aligned micropatterns. Micropatterns were then used as moulds to fabricate scaffolds of biocompatible polymer for cell culture and microwells for cell pattering or bioanalytics. The favourable cultivation of human epithelial cells on PDMS microwells has demonstrated the potential applications as tissue engineering scaffolds or cell-based biosensors. It is expected that, by proper surface modification, such as coating of adhesion protein or growth factors to the scaffold surface, it is possible to further enhance the biocompatibility, cell-substrate interactions, cell adhesion and cell proliferation. Further research will also be carried out by incorporating microspheres in microwells to obtain bead-based assays for multi-analytes detection achieving one million detection sites per square centimeter. The proposed technique of micromolding various microstructures offers a rapid, simple, economical, and versatile alternative to the existing microfabrication techniques, and will have impact on the technology of advance materials patterning and lithography, bioengineering, and biomolecular analytics.

Acknowledgements

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