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Abstract:

We fabricated adjacent micropillar arrays with different heights using different materials. Masters are obtained by using a two-step photolithography technique. A thin layer of SU8-3005 resist was spin coated on a chromium mask, followed by a front side exposure with another assistance mask, resulting in a step resist profile. Then, a thin layer of SU8-3010 resist was spin-coated again on the mask, followed by a back side exposure. The master patterns which consist of adjacent pillar arrays of different heights were replicated into polydimethylsiloxane (PDMS) by soft lithography. The PDMS replicas were then used as molds for casting or hot embossing, resulted in final pillars arrays in PDMS, poly lactic-co-glycolic acid (PLGA) and flexestene thermoplastic elastomer. Such substrates made of different materials were used to evaluate the surface stiffness dependent cell migration of NIH 3T3 cells. Our results show that the cells were sensitive to the height of PDMS pillars, due to their comparable Young's module, and that the cells were preferentially localized on the stiffer surfaces. However, no such effect was observed when the cells were placed on the PLGA substrate because of the excessive rigidity of the PLGA pillars.

Keywords: Micro Pillars Array, Cell Culture, Cell-Materials Interaction

1. Introduction

Cell adhesion and cell migration are important in cell biology and biomedical studies [1, 2]. For in-vitro studies, the mechanic stiffness of the substrate has to be adjusted to the requested cell functions [3, 4]. Ideally, the mechanic properties of the fabricated substrate should be as close as possible to that of the extracellular matrix (ECM) of the in-vivo cellular microenvironment [5, 6]. Therefore, it is interesting to study cell adhesion and cell migration behaviors on artificial ECM of different stiffness. Previously, the most of the investigations were performed on substrates with uniform stiffness, with or without surface patterning, which cannot be used to evaluate the cell migration behaviors [7-9]. In this work, we aimed at observation of preferential cell adhesion on artificial ECM having adjacent area of different stiffness. Such an observation should be necessary for more clear understanding of cell adhesion, spreading and migration. We have chosen a particular type of artificial ECM made of adjacent micropillar arrays with different heights which can be easily fabricated and used for quantitative assessment.

Micropillar arrays are now frequently used to obtain substrate of different stiffness and to determine the mechanic properties of the cells by changing the height of the pillars and measuring the deflection of the pillars [10, 11]. Previous studies also demonstrated that cells are very sensitive to the stiffness of the pillars which is determined by both the material Young's module and the size/height of the pillars. However, such a sensibility is clearly cell-type and material stiffness range dependent. The challenge was then fabricating the adjacent micropillar arrays of different height using materials of different Young's modules and demonstrating the preferential cell attachment on micropillars with more appropriate stiffness.

2. Fabrication Protocol of Micropillar Arrays

2.1. Materials

Blank chromium mask (Nanofilm Inc) and resist developer AZ726 (AZ Electronic Materials) were purchased from Cipec (France). Chrome Etch N°1 was purchased from Technic Inc. (France). SU8 photoresists (SU8-3005 & SU8-3010) and SU8 developer were obtained from CTS (France). Polydimethylsiloxane (PDMS) was purchased from Eleco-EFD (France). Polylactic-co-glycolic acid (PLGA) was obtained from Sigma-Aldrich (France). Styrenics block copolymer, a typical flexestene thermoplastic elastomer (T-flex), was supplied by Laboratoire de Photonique et de Nanostructures, UPR 20 CNRS, Marcoussis, France.

2.2. Fabrication of the mold

Figure 1 shows a process flow to fabricate molds of micropillars with two different heights. Firstly, periodic holes were patterned on the blank chrome mask pre-coated with a photoresist layer (AZ 1518) by using a micro-pattern generator (μPG101). After Cr etching and the resist removal, the Cr mask was spin-coated with a 4-μm thick negative resist layer (SU8-3005). This resist layer was then UV exposed from the front side with another mask, resulting in a Cr mask partially covered by the SU8 resist (exposed). After development, a 9-μm thick negative resist layer (SU8-3010) was spin-coated on, following by the back-side UV exposure. As results, two adjacent pillar arrays of 4 μm and 9 μm heights were obtained, as shown by the SEM picture in Figure 1.

2.3. Fabrication of the substrates

The Cr mask with SU8 pillars of different heights was used as mold to produce different substrates for cell culture (figure 2). To facilitate the replica, the mold patterns were first replicated in to PDMS. After surface treatment of oxygen plasma for 90 s and trimethylsilanechloride (TMCS) evaporation for 3 min, a mixture of PDMS pre-polymer and crosslink agent at ratio of 10:1 was poured on the mold and cured at 80°C for 2 h. After separation, the PDMS mold was treated by oxygen plasma for 90 s and TMCS evaporation for 3 min. The second replication was obtained with three different types of polymers, PDMS by casting, PLGA and T-flex by hot embossing, using the same PDMS mold. For PDMS, the replica was obtained by casting PDMS against the PDMS mold and curing at 80°C for 2 h. For PLGA and T-flex, the samples were embossed for 10 min with a pair of on hot plates at 70°C and 150°C respectively. Figure 2 shows SEM images of two replicated pillar arrays of 7 μm heights in PLGA and T-flex, respectively. Apparently, the shape of PLGA and T-flex pillars; both obtained by hot embossing, are different. This is because of the fact that PLGA is typical thermoplastic polymer while T-flex is a thermoplastic elastomer, which have different responses to the mechanical stress induced by hot embossing. After hot embossing, the released PLGA pillars can more easily keep the shape than that of T-flex which exhibited a rounded top as well as a smaller pillar height.

3. Cell Culture

3.1. Cells and reagents

NIH 3T3 was obtained from ATCC Co. (France). Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), penicillin, streptomycin, trypsin, fibronectin and Dulbecco's Phosphate Buffered Saline (DPBS) were purchased from Gibco (Life Science, France). 4',6-diamidino-2-phenylindole (DAPI), Phalloidin and Fluorescein isothiocyanate (FITC) were purchased from Sigma-Aldrich (France).

3.2. Cell culture and staining

After rinsed with isopropanol (IPA), dried with nitrogen (N_2) and put in vacuum chamber overnight, the fabricated pillar substrates were exposed to UV light for 1 h for sterilization. Subsequently, the samples were coated with fibronectin by incubation for 2 h at 25°C in a fume hood, in order to improve the adhesion of cells on pillars. Meanwhile, cells were prepared in growth media (DMEM with 10% FBS, 1% penicillin/streptomycin) and trypsinized, centrifuged and re-suspended with cell density ~10^5 cell/mL. 50-µL solution (cells in media) was dropped on each substrate. After incubation for 1 h for cell attachment, new culture media were added and the samples were placed in an incubator (37 ℃, 5% CO_2).

After 2/4-days culture, the cells were fixed with 4% paraformaldehyde (PFA in DPBS) for 10 min and stained with DAPI (600 nM in DPBS), Phallodin-FITC (20 µM in DPBS) for 20 min.

4. Results and Discussion

Figure 3 shows fluorescence images of NIH3T3 cells on a flat surface (A) and a SU8 pillar array of 30 µm pitch size and 10 µm spacing (B). On the flat surface, the cell density is higher than that on the pillar substrate. The cells also spread over a relative large area on the flat surface. On the pillar substrate, fewer cells are observed due to limited proliferation and they also fell into the gap area of the pillars, showing elongated fibers. This is because of the large distance between the pillars but limited size of the pillars, resulting in a low proliferation rate. Interestingly, the nuclei of the cell fell into the gap between the pillars are significantly deformed without external force.

When the cells are placed on the denser pillar arrays, they have different behaviors. Figure 4 shows fluorescence images of NIH 3T3 cells after incubation for 4 days on PDMS (A) and PLGA (B) pillars of 5 μm spacing, respectively. Here, the distance between pillars is significantly smaller than that of the cell sizes so that cells can stay comfortably on the top and spread over several pillars. Now, the pillar substrate can be equivalently considered as a flat substrate with a lower effective stiffness. To assess such an effective stiffness, we may first calculate the sprint constant of a single pillar,

$$k = \frac{3}{4}\pi E \frac{r^4}{H^3} \tag{1}$$

where E is the Young modulus of the material, r and H are the radius and height of the pillars, respectively [12]. Then, the equivalent Young modulus of the surface composed of dense pillar arrays can be obtained by [13],

$$E_e = \frac{9k}{4\pi r} \tag{2}$$

The Young's module of PDMS and PLGA is in the order of 1 MPa [14] and 1 GPa [15], respectively. Assuming a pillar diameter of 2 μm and a pillar height of 4 μm (9 μm), we deduced an equivalent Young modulus of 2.3 kPa (26.4 kPa) for PDMS and 2.3 MPa (26.4 MPa) for PLGA pillars. Since the Young's module of the PDMS pillar surface is comparable to that of migrating NIH 3T3 cells (3-12 kPa) [16], NIH 3T3 cells should be more sensitively to the variation of the stiffness of the PDMS pillars. Indeed, when cells were placed on PDMS pillars of different heights, they preferentially localized on the stiffer (smaller height) pillar areas (Fig. 4A). Coherently, much more cells were found on PLGA pillars but they showed no preferential attachment since the effective Young's modules of the PLGA pillars are both excessively larger than that of the cells. Finally, the Young modulus of T-flex is comparable to that of PDMS so that we observed quite similar phenomena on both types of pillar substrates.

5. Conclusion

Adjacent micropillar arrays of different heights were fabricated by casting or hot embossing in PDMS, PLGA and T-Flex, resulting culture substrates of different effective stiffness, ranging from 10 kPa to 10 MPa. Our results show that NIH 3T3 cells are sensitive to the height of PDMS pillars due to their comparable Young's modules and that these cells are preferentially localized on the stiffer pillar area. However, no such effect has been observed when

the same cells are cultured on PLGA pillars of different heights because of the much larger effective stiffness of the PLGA pillars.

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Figure 1. Schematic process flow of SU8 mold fabrication and SEM picture of the mold.

Figure 2. (A) Schematic process flow of pillar array replication by casting and hot embossing, respectively. (B, C) SEM pictures of the replicated PLGA and T-Flex micropillars.

Figure 3. Fluorescence images of NIH 3T3 cells after incubation for 2 days on silicon wafer (A), and a SU8 pillar array of 30μm pitch size and 10μm spacing (B).

Figure 4. Fluorescence images of NIH 3T3 cells after incubation for 4 days on PDMS (A) and PLGA (B) pillars of 5 μm spacing, respectively.

Figure 1

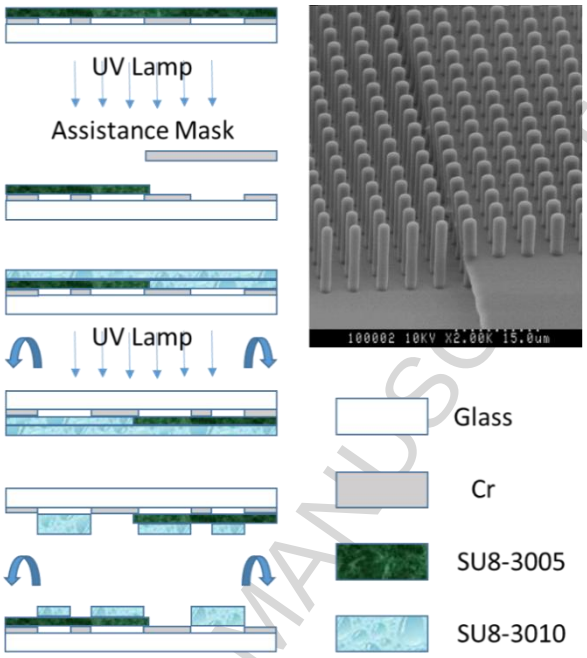


Figure 2

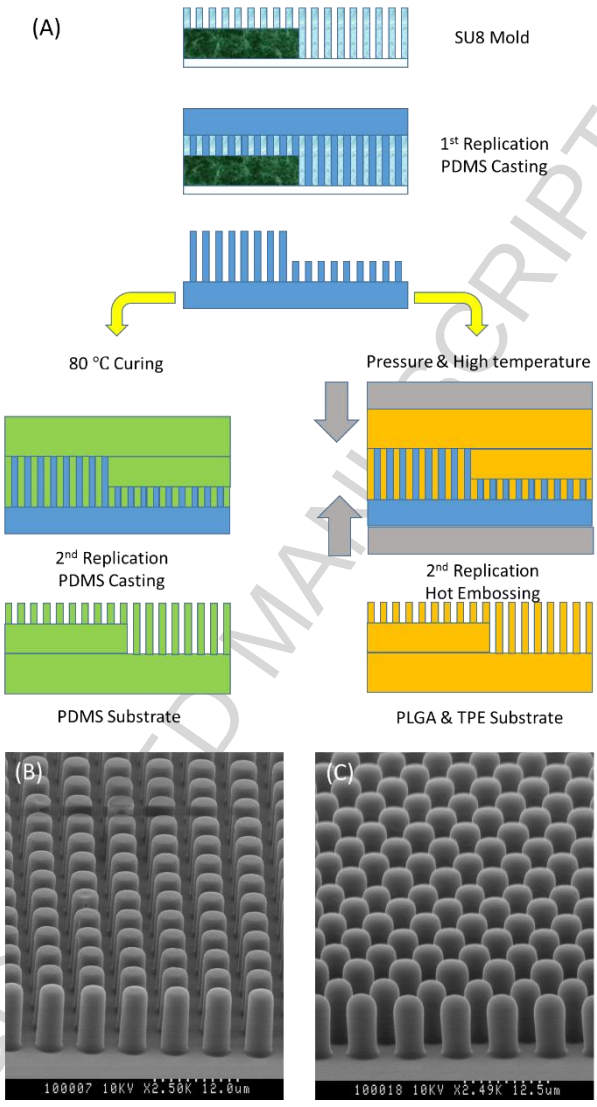


Figure 3

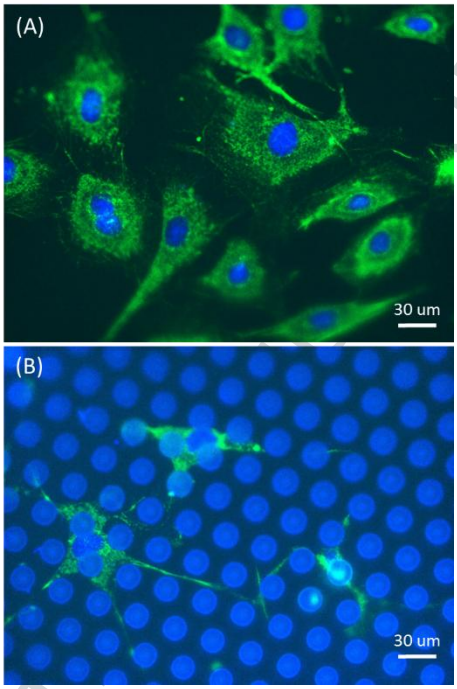
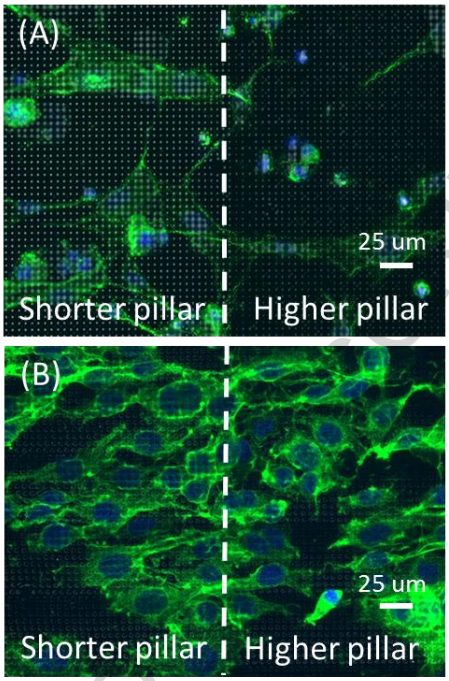
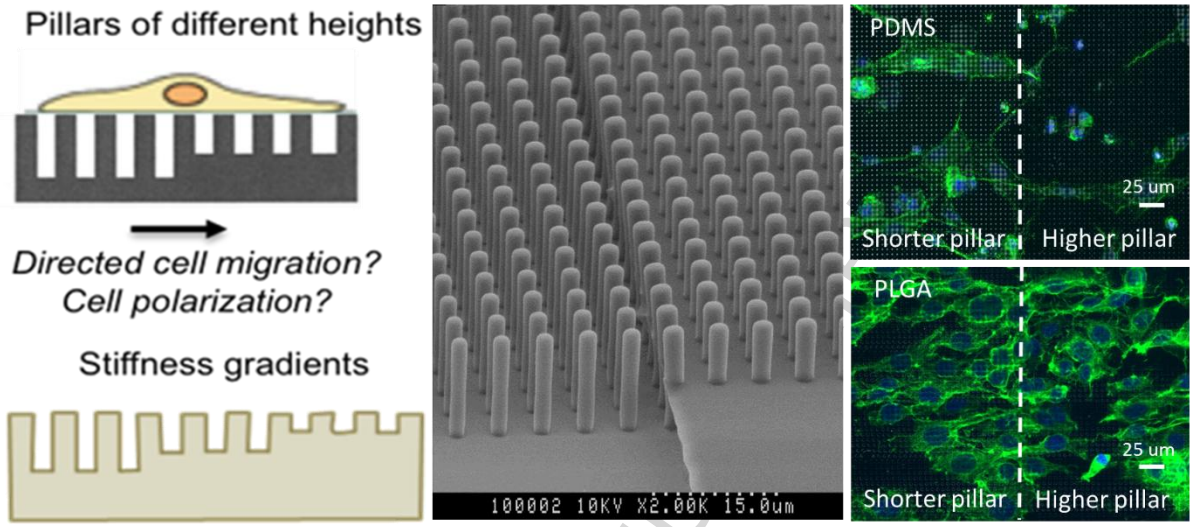


Figure 4





Graphical abstract

Highlights:

1. A two-step photolithography is used to fabricate masters of adjacent pillar arrays and PDMS molds
2. Adjacent PDMS micropillar arrays of different heights are obtained by casting of PMDS.
3. Adjacent PLGA and T-Flex micropillar arrays of different heights are obtained by hot embossing.
4. NIH 3T3 cells are sensitive to the height of the pillars when their Young's modules are comparable.