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Simple fabrication of functionalized surface with polyethylene glycol microstructure and glycidyl methacrylate moiety for the selective immobilization of proteins and cells

Hwan-Moon Song and Chang-Soo Lee†

Department of Chemical Engineering, Chungnam National University, 220 Gung-dong, Yu-Seong gu, Daejeon 305-764, Korea
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Abstract−This study reported simple surface modification for the immobilization of biomolecules such as proteins and cells onto desired area at micron-scale level. First, thin film composed of glycidyl methacrylate (GMA) was prepared by UV-photopolymerization. Then, the polyethylene glycol (PEG) microstructures which played a role in the prevention of nonspecific binding of biomolecules were fabricated by using micromolding in capillaries (MIMIC). Thus, we could easily obtain an orthogonal surface having biomolecular attraction and repulsion areas. In addition, we could control of the height of prepared PEG microstructures with spin coating or not. For the investigation of feasibility of biomolecule patterning onto the functionalized surface, FITC-BSA and HEK 293 were examined as representative biomolecule models. A functionalized surface with GMA promotes the strong adhesion of biomolecules, and PEG microstructures located on the background prevent nonspecific binding of biomolecules at micron-scale level. The orthogonal difference in surface functionality showed strong possibility of simple patterning of biomolecules. In addition, the proposed method could easily control the size, shape, and height of patterns. It will be useful platform technology for the construction of a biomolecule array.

Key words: Surface Modification, Protein Patterning, Cell Patterning, Micromolding in Capillaries, PEG

INTRODUCTION

The control of immobilization of biomolecules onto a desired area is an important technique in various applications such as biosensors, biochips, and microdevices because it can perform high throughput analysis for a short time compared with conventional methods [1-3]. However, selective immobilization of biomolecules ("patterning") is difficult to achieve because of nonspecific binding of biomolecules onto fabricated microdevices and the limitation of biomolecules' stability for long-term study. Therefore, the fabrication of a unique surface having the capability of prevention of the non-specific binding and high binding performance of biomolecules is the most important tool in various fields [4,5].

Recently, polyethylene glycolated polymers have been a focus to create useful surfaces because of their biocompatibility and nonfouling materials [6-8]. There have been several reports for the fabrication methods with PEG polymers including photolithography [9,10] and stencil [11]. However, these require several steps, high process cost, expensive equipment, and difficult handling. Especially, the use of photoresist is a detrimental effect of the inherent property of proteins or cells, which results in the loss of activity or decrease of their functional activities under the harsh photolithographic conditions.

To solve the above issues, our group has proposed a micromolding in capillaries (MIMIC) method with the photopolymerization of polyethylene glycol dimethacrylate, which shows the feasibility of selective biomolecule patterning. This method produces reproducible features corresponding to the PDMS mold and provides a physical, chemical, and biological barrier without the loss of activity of biomolecules [12]. This study introduces a simple biomolecule-patterning fabricated by soft lithographic technique of MIMIC in which the PEG microstructures using rapid photopolymerization strongly cross-linked with previously GMA coated surface. While the epoxide regions allow selective binding of biomolecules with strong covalent bonding, PEG microstructures provide biological barriers that prevent nonspecific biomolecule binding. Thus, the orthogonally different surface having biomolecule repellent and adhering regions can be easily produced by combined surface modification with MIMIC. Our proposed methods can be useful for the fabrication of biomedical microdevices via a simple process.

EXPERIMENTAL

1. Materials

Glycidyl methacrylate, 3-(trimethoxysilyl) propyl methacrylate, poly(ethylene glycol) dimethacrylate (M_w=330), phosphate-buffered saline, fluorescein isothiocyanate - bovine serum albumin (FITC-BSA), and cell culture medium containing DMEM, fatal bovine serum, and antibiotics were purchased from Sigma-Aldrich. Poly(dimethylsiloxane) (PDMS) (Sylgard 184 elastomer) was purchased from Dow Corning and 2-hydroxy-2-methyl-1-phenyl propanone (photoinitiator) was obtained from Ciba Specialty Chemicals (Switzerland). Purified water (Millipore) was used throughout process.

2. Fabrication of Functionalized Surface Having PEG and GMA Region

As shown in Fig. 1, the glass substrate (2 cm×2 cm) was sequentially washed with acetone, ethanol, and distilled water. The cleaned glass substrate was immersed in piranha solution and treated with O_2 plasma. First, the substrate was coated with 3-(trimethoxysilyl)

†To whom correspondence should be addressed.
E-mail: rhadum@cnu.ac.kr

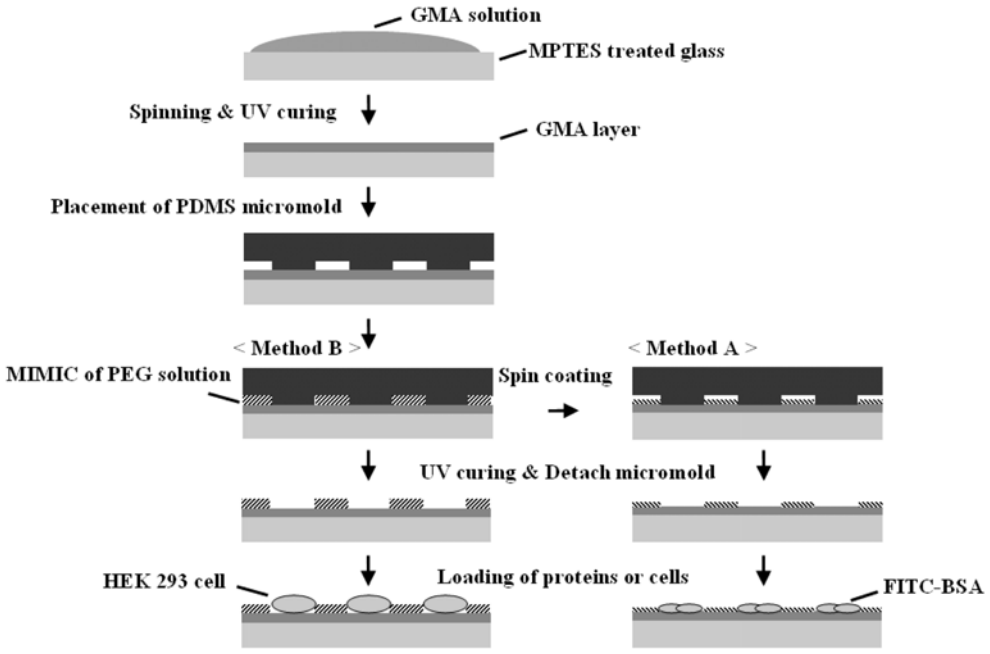

Fig. 1. Schematic diagram of selective biomolecules patterning on fabricated surface. Method B was applied to immobilize the HEK 293 cell and method A showed selective patterning method of FITC-BSA.

propyl methacrylate (MPTES) solution (2%, methanol) [13]. The surface was then spin-coated with glycidyl methacrylate (GMA) containing 1% (v/v) photoinitiator. It was rapidly polymerized by UV irradiation at 365 nm for 10 min [14]. PDMS micromold placed on the cured substrate and micromolding in capillary (MIMIC) performed with poly(ethylene glycol) dimethacrylate (PEG-DMA) [15]. In the MIMIC approach, the PDMS molds having various shapes such as circle, square, and triangle and different sizes such as 50 μm, 100 μm, and 200 μm were used.

Two kinds of methods of PEG micromolding were designed for the control of height of PEG microstructures. In first method (A), in order to fabricate lower height of PEG microstructures, PEG solution was slowly injected along the microchannels with the capillary force and it was spin-coated at 3,000 rpm for 15 seconds to make the flattened morphology of PEG microstructures. In the case of the B method, the simple loading of PEG solution was flowed along the PDMS micromold without spin-coating, which produced higher height of PEG microstructures. After the loading of PEG into the micromold and photopolymerization of the PEG, the UV-cured microstructures of PEG were peeled from the PDMS mold and it was stored at 4 °C. The morphology of the fabricated substrate was monitored by atomic force microscopy (AFM) and scanning electron microscopy (SEM).

3. Protein Patterning on Fabricated Surface

An FITC-BSA solution having various concentrations (0.001-100 μg/ml) was applied to the fabricated surface. Each of FITC-BSA samples was carefully loaded on the fabricated substrate (method A) and then placed in a dark region immediately to avoid exposure to an external light source for 30 min. Thereafter, unconjugated proteins were washed out by PBS buffer and dried with nitrogen gas. All these steps were performed in sterilized conditions to prevent biological contamination. Finally, the images of protein patterns were captured by inverted fluorescence microscopy and analyzed with ImageJ (http://rsb.info.nih.gov/ij/).

4. Mammalian Cell Patterning

The epithelial kidney cell (HEK 293) was examined as a representative animal cell in our study. The mammalian cells were patterned on the sterilized microstructures fabricated by method B. The HEK 293 cells (10^5 cells/patterned structure) were loaded on each microstructure and then incubated for 30 min. Next, the patterned cells on each substrate were cultivated in the incubator supplemented with 5% CO_2 at 37 °C. The images of adherent cell were also captured by optical microscopy. The schematic diagram of overall patterning procedures of protein or mammalian cell is shown in Fig. 1.

5. Measurements

Optical and fluorescence images were obtained by microscope (TE-2000U, Nikon) using ImageJ software. Morphological analysis of fabricated microstructures was analyzed with atomic force microscopy (AFM) (XE-100, PSIA, Korea) by using the XEI analysis program and scanning electron microscopy (SEM) (JSM-7000F, JEOL, Japan). The water contact angle of each step was measured by contact angle analyzer (KRUSS, PSA100, Germany). The values of contact angles were calculated by measuring for several times.

RESULTS AND DISCUSSION

1. Preparation of PEG Microstructures on GMA-Coated Surface

In method A (Fig. 1), the height of PEG microstructures could be freely controlled by the modulation of spinning condition. Fig. 2(a) indicates that the AFM image of structural height between PEG microstructure and GMA surface was about 40 nm. However, the boundary line was measured from 700 nm to 1 μm (Fig. 2(b)). This might be attributed to the capillary rise of PEGDMA solution in

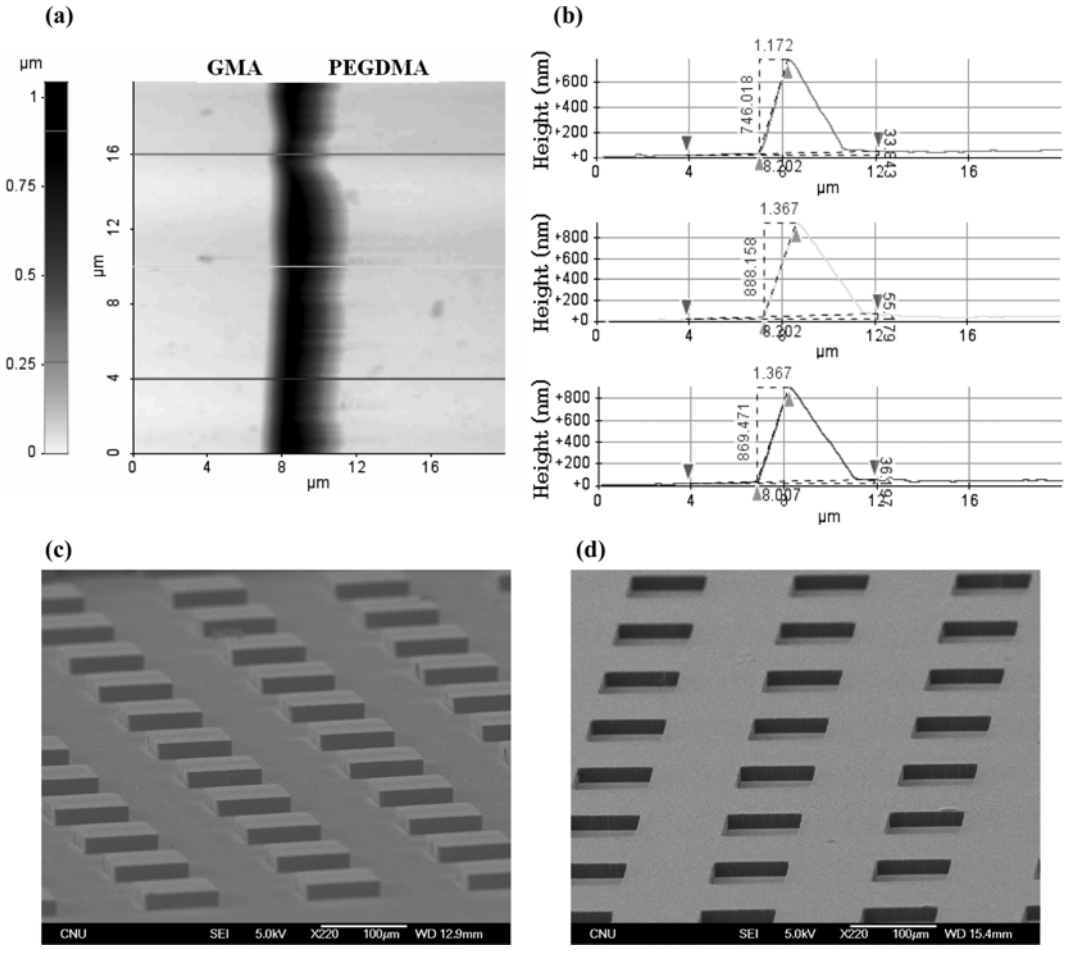

Fig. 2. The morphological analysis of substrates with atomic force microscopy (AFM) and scanning electron microscopy (SEM). (a) Two-dimensional AFM image between GMA coated surface and PEG microstructure (method A). The scanning region was 20 μm× 20 μm. (b) line profile of cross-section between GMA surface and PEG microstructure. The SEM images of (c) PDMS micromold and (d) PEG microstructures fabricated by method B.

the PDMS micromold [16]. These PEG microstructures were stable against a biological environment such as cell medium and PBS buffer for several weeks. The above result proved that PEG microstructures were strongly attached onto the GMA surface and had structural resistance against biological environment.

The SEM images of PDMS micromold and fabricated PEG microstructure are shown in Fig. 2(c) and 2(d). The PEG microstructure was fabricated from method B. As expected, PEG microstructures on GMA surface did not degrade and shrink after the removal of PDMS micromold, which also indicated that our proposed method performed correctly. The measured width (200 μm) and depth (20 μm) were corresponding to PDMS micromold using the α-step analyzer (data not shown). Thus, we could confirm that method B for the fabrication of microstructures resulted in the correct formation of replica and could be applied to fabricate various shapes and sizes of microstructures.

2. Contact Angle of the Fabricated Microstructures Surface

The analysis of contact angle on different substrates indirectly confirmed the surface modification because all treated surfaces have different properties of wetting. As shown in Fig. 3, all substrates in accordance with surface treatment had different values of water contact angle. In detail, contact angles of MPTES and GMA coated surface were measured to 51.3±1.3° and 37.8±1.2°, respectively. Those values were well matched with previous results [17,18]. It indicated that the MPETS solution was covalently bonded as silanization mechanism with hydroxyl group of O_2 plasma-treated surface and then acrylate group of MPTES was able to link with GMA solution through UV exposure.

Because the PEGDMA microstructure gave more hydrophobic property resulting from the high content of air trapping, the surface of PEGDMA microstructures (55.5±4.5° from method A and 63.3± 3.9° from method B) showed larger static contact angle than those of bare glass (14.4±1.4°) and PEG thin film (25.5±1.2°) [19]. The air space between the microstructures resists the wetting of dropped water solution. Thus, the wetting property of water critically depends on the geometrical effect of fabricated microstructure size, vertical height, and shapes.

3. Quantitative Patterning of Protein on the Prepared Surface

Fig. 4 clearly shows that patterning of FITC-BSA was successfully performed on the prepared substrate. Our proposed method A provided well ordered patterns with various shapes such as circle (Fig. 4(a)), triangle (Fig. 4(b)), and square (Fig. 4(c)) at micron scale.

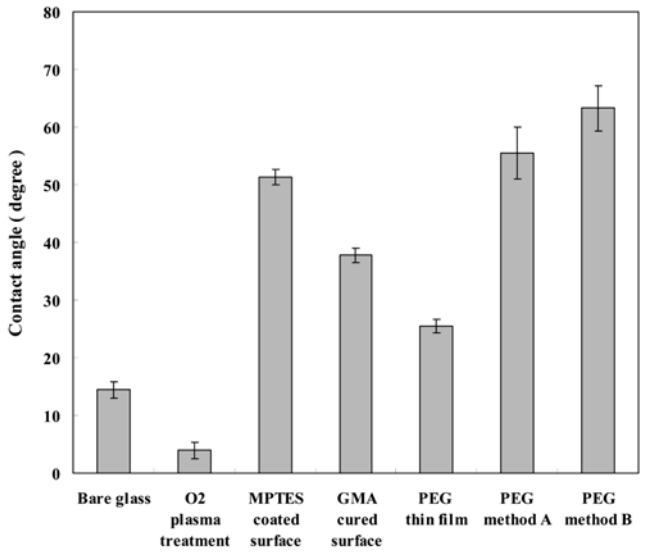

Fig. 3. The water contact angles on various surfaces. Average values and standard deviations were obtained by several separate measurements of each substrate. Bare glass was evaluated after the cleaning condition and O_2 plasma treatment. MPTES coated surface was prepared by silanization process after the treatment of O_2 plasma. GMA surface on MPTES-treated surface was examined after the UV exposure. The various PEG surfaces including PEG thin film and two kinds of PEG microstructures were prepared on GMA surface through the UV irradiation. Furthermore, PEG microstructures were evaluated as described by experimental method A and method B.

As shown in Fig. 4(d), highly ordered two dimensional protein patterns showed high signal-to-noise ratio (pattern intensity/background intensity: 47.2±7.3/2.3±2.1).

The results confirmed that PEG microstructures provided a biological barrier for the prevention of nonspecific binding of proteins onto background region, and the epoxide group of the GMA region was selectively conjugated with BSA proteins. The patterned proteins were stably maintained on the GMA region after several washings because proteins were stably retained through the formation of covalent binding between surface and proteins. Thus, it could be a good matrix for selective immobilization of proteins with flexible control of size, shape, and height at microlevel.

Finally, we investigated the feasibility of the quantitative patterning of proteins with various concentrations of FITC-BSA on the surface fabricated by method A. Fig. 5 shows the limit of detection (LOD) was about 1 ng/ml and maximum concentration of protein was 100 μg/ml. As expected, a fluorescence signal of patterned proteins could be detected at 15.4 pM of protein in consideration of BSA molecular weight (65,000 Da). As a result, the fluorescence intensity was increased according to increasing the FITC-BSA concentration and became saturation above 25 μg/ml. It indicated that our proposed method could be applied to protein based biosensors in microdevices.

3. Mammalian Cell (HEK 293cell) Patterning

For the investigation of versatile applications, cell patterning was carried out with the HEK 293 mammalian cell line. Fig. 6(a) represents that cell patterning was correctly performed on the fabricated substrate (method B). This result also showed that the PEG micro-

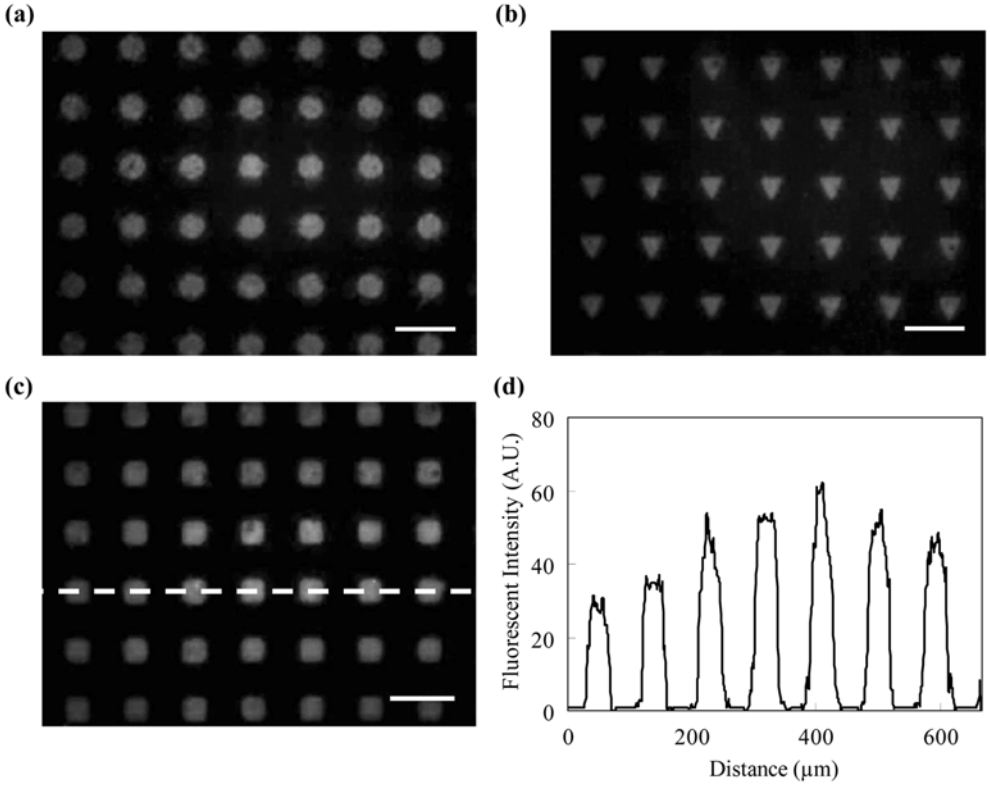

Fig. 4. Fluorescence images of patterned FITC-BSA with (a) circle, (b) triangle, and (c) square patterns. The scale bar indicates 100 μm. (d) line profile of fluorescence intensity with high ratio of signal to noise. The concentration of FITC-BSA was 1 μg/ml.

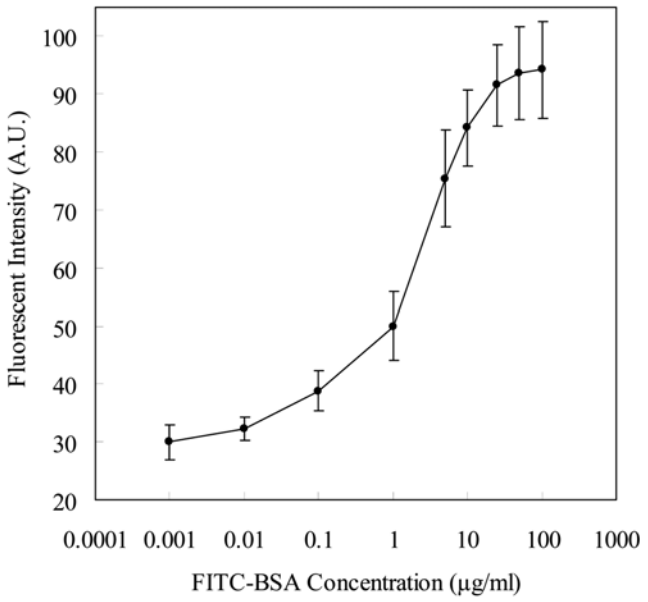

Fig. 5. Plot of the fluorescent intensity versus different concentrations of FITC-BSA. All patterns were square shape of 50 μm×50 μm. The signal intensity of all samples was measured at 500 ms exposure time. These results were obtained from the average value of 125 squares per patterned region.

structure prevented nonspecific binding of animal cells and only GMA surface was attached with HEK 293 cell. Because most of the cells have several membrane proteins on their outer membrane, the proteins can be covalently conjugated with epoxide group on the GMA surface. Additionally, HEK293 cells in GMA region could survive for 1 week (Fig. 6(b)). Compared with the cell morphology of cells on a conventional cell culture plate, a spherical shape of adhesive cells on the fabricated surface was obtained, which indirectly suggested that the covalent binding of cells onto the surface rendered more restricted conditions although cell patterning was successfully achieved. The fabricated substrate, however, could be applied to the development of cell-based microdevices without nonspecific binding of cells during several days.

CONCLUSION

We demonstrated a simple method for the selective immobilization of proteins and cells on the functionalized surface using MIMIC technique. The fabricated PEG microstructures could be freely manipulated and provide a biological barrier for the prevention of nonspecific binding of proteins and cells. In addition, the exposed GMA region rendered an efficient binding region of biomolecules with long-term stability of biomolecule patterns. This method has the strong advantage of easy control of shapes, sizes, and heights of desired patterns at the microlevel.

Therefore, this approach could be applied for a universal platform of bioassay, biosensor, cell based microsystems and combined microfluidic systems without a complicated process.

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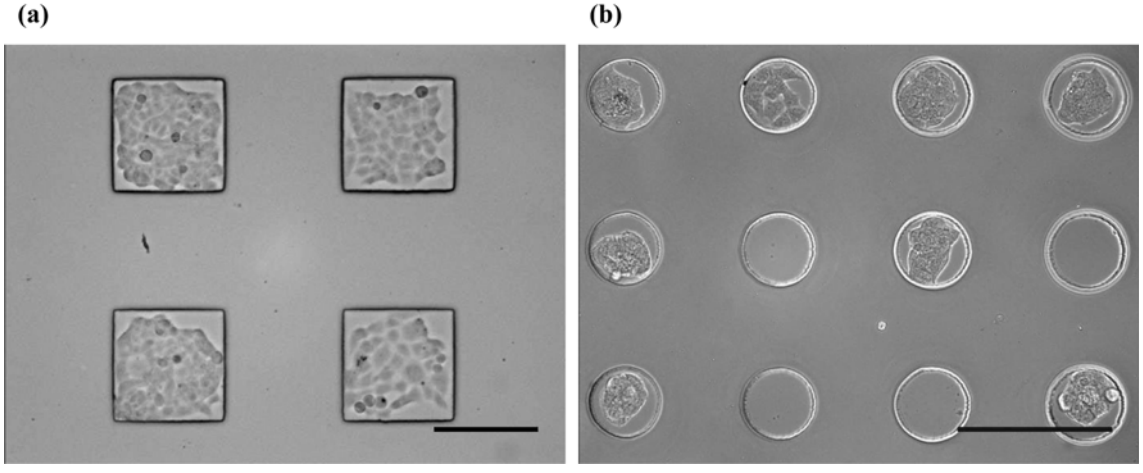

Fig. 6. Optical images of pattern of HEK293 cells. (a) the cell images after 24 hours (b) cell images after 72 hours. The scale bar represents 200 μm and 100 μm, respectively.

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