Fabrication of micro-structured scaffold using self-assembled particles and effects of surface geometries on cell adhesion

Iwori TAKEDA*, Shogo SERIZAWA* and Arata KANEKO*
*Tokyo Metropolitan University
6-6 Asahigaoka, Hino, Tokyo 164-0001, Japan
E-mail: takeda-iwori@ed.tmu.ac.jp

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Abstract

Controlling of cell location is needed for some cellular applications like drug screening. Micro/nano-structured surface are used for controlling of cell location without using any chemical agents. An average roughness is of interest for investigating an effect on location of cell adhesion. However, some studies have indicated different results about cell adhesion even though using same kinds of cell line, material properties of scaffolds, and geometrical properties of scaffolds. Those studies have investigated an effect of average roughness only. An average roughness, therefore, is not sufficient for classifying the structured surface. In addition, the structured surfaces have no geometric regularity typically. To resolve these problems, the authors employed regularly arranged surface for cell culture scaffold and investigated effects of an average roughness, skewness, and kurtosis on cell adhesion. The authors used self-assembled SiO₂ particles as a mask of reactive ion etching of Si wafer for fabricating micro-structured substrate. Then, geometric transferring technique of polydimethylsiloxane is used for fabricating cell culture scaffolds. An average roughness, skewness, and kurtosis of the scaffolds can be controlled by changing RF power and etching time. The structure that has negative skewness improves cell adhesion was found. It can be seen that Rsk of surface works as an important factor for cell adhesion.

Key words : Cell adhesion, Particle, Reactive ion etching, Skewness, Kurtosis

1. Introduction

A development of cell patterning technique is required for an application of bio-chemical sensing, cellular analysis, and bio-mechanical actuator (Nagai, et al., 2014). Although it is found that micro/nano-scale topography of scaffold affects cell adhesion, its mechanism has not been clarified. Most researchers suggested that effects of surface roughness are an important parameter to dominate cell adhesion. For example, mesenchymal stem cells preferentially adhere on a nano-structured surface with specific surface roughness (Ra) ranging from 2.1 to 3.1 μm (Faia-Torres, et al., 2014). It has been reported that a surface morphology with Ra of ~11 nm also enhanced adhesion of human fetal osteoblast cells (Yang, et al., 2013). Interestingly, there were different properties in an effect of surface roughness on cell adhesion, although they utilized same type of cells and similar material of scaffold (Fan, et al., 2002; Khan, et al., 2005). Fan et al. shows that highest viability of neural cells on Si wafer is indicated in the case of Ra = 25 nm. Khan et al., however, shows that highest viability of neural cells on Si wafer is indicated in the case of Ra = 60-70 nm. These results of previous researches summarized that surface roughness (Ra) is not necessarily dominant in cell adhesion, although surface morphology is a key factor. Cell adhesion involves some processes such as protein adsorption, a change of cellular morphology, and filipodium extension. Therefore, it is considered that they are attributed to sharpness (skewness, kurtosis) and spatial frequency of surface asperities as well as surface roughness (Ra) representing average height. This present study is also intended to investigate an effect of surface geometrical features on cell adhesion. The authors have demonstrated a technique for a fabrication of structured surface by self-assembled particles (Nishio, et al., 2014a; 2014b). This self-assembly technique easily provides specific surface asperities on hexagonally arranged fine particles. It is also demonstrated that assembled particles works as a cell culture scaffolds (Balmert and Little, 2012; Kaneko, et al., 2012; Kruss, et al., 2010; Sugihara and Kaneko, 2013; Takeda, et al., 2012; Takeda, et al., 2015a;
Takeda, et al., 2015b). In this present study, some surface with different geometrical feature (roughness, skewness ($R_{sk}$) and kurtosis ($R_{ku}$)) were prepared by a combination of the above described self-assembly technique with dry etching and molding.

2. Surface parameters

As described above, surface roughness ($R_a$), skewness ($R_{sk}$) and kurtosis ($R_{ku}$) are employed to classify surface geometries of scaffold. They are determined by the following equations (1) – (3) and Fig. 1, where $R_q$ means root mean square $R_a$. Skewness ($R_{sk}$) indicates symmetry in peaks and valley of surface asperities, so that the shaper peaks and the gentle valley makes skewness larger. When a surface has symmetric height distributions, the value of $R_{sk}$ is 0. Meanwhile, kurtosis ($R_{ku}$) represents a sharpness of height distribution. The sharper peaks and valleys of surface make the value of $R_{ku}$ larger, while a rectangular-like cross-section decreases that of $R_{ku}$. When a height of surface indicates normally-distribution, the value of $R_{ku}$ is 3.

$$R_a = \frac{1}{L} \int_0^L f(x) \, dx$$

$$R_{sk} = \frac{1}{R_q^3} \left\{ \frac{1}{L} \int_0^L f^3(x) \, dx \right\}$$

$$R_{ku} = \frac{1}{R_q^4} \left\{ \frac{1}{L} \int_0^L f^4(x) \, dx \right\}$$

3. Materials and Methods

The authors employed self-assembly of fine particles techniques, reactive ion etching (RIE) and molding to fabricate structured surfaces for cell culture scaffold. Figure 2 shows a schematic illustration of surface preparation. This process consists of three steps.

A Si (001) wafer (Matsuzaki Seisakusyo Co., Ltd.) or a glass plate (Matsunami Glass Ind. Ltd.) is previously treated in piranha solution (1:3 solution of hydrogen peroxide and sulfuric acid) to be hydrophilic (water contact angle < 5°). These substrates are processed by micro-contact print, so that line patterns of octadecyltrichlorosilane (OTS, Thermo Fisher Scientific Inc.) are transferred onto the substrate. The substrate have an array of hydrophobic and hydrophilic lines, because the surface of OTS indicate hydrophobic (water contact angle = 105°). The patterned substrate was subsequently drawn up from water suspension of SiO$_2$ particles (1 μm diameter; Hipresica, Ube Exsymo Co., Ltd.). The SiO$_2$ particles are autonomously assembled on only the hydrophilic regions, as they closely packed by meniscus force (Kanamori, et al., 2008; Kumar, et al., 1994). The self-assembled particles are used as an etching mask (Gianetta, et al., 2013; Tanaka, et al., 2012). The substrate is locally etched off in the gap between assembled particles, so that arrays of tapered micro-pillars are gradually fabricated. The geometrical features of etched surface can be controlled by etching conditions (RF power and etching time). The particles are completely removed by ultrasonic cleaning after etching process. The etched surface is casted by a pre-polymer of polydimethylsiloxane (PDMS, SYLGARD 184, Sigma-Aldrich Co., LLC.), so that the micro-structured PDMS is fabricated. A micro-structured PDMS is applied to cell scaffold, while another is utilized for a second master. The latter process provides an inverted structure of PDMS (Shao, et al., 2012). The authors prepared 6 kinds of PDMS scaffolds. All of scaffolds were finally coated by polystyrene thin film.

3. Results and Discussion

3.1 Surface properties of structured substrates

Figure 3 shows SEM image of typical example of self-assembled micro-particles on a Si wafer. The hexagonally packed particles are aligned in a line-and-space pattern. The line width and spacing of micro-structured particles were about 30-40 μm and 70-60 μm, respectively. These assembled particles are mainly formed in a monolayer, so that they could be applied a mask of dry etching. Figure 4 shows a SEM image of Si wafer after dry etching with CF$_4$ for 10 min. There are tapered micro-pillars which are regularly arranged. The distance between micro-pillars is almost corresponded to that of the diameter of particles (1 μm). The height of micro-pillar is about 200 nm. The geometry of these structures was transferred by PDMS.

Figure 5 shows AFM images of typical PDMS surfaces after molding. Scaffolds $a$, $c$, and $e$ have hexagonally
aligned micro-holes, while Scaffolds \( b, d, \) and \( f \) have an array of pillar-like structures. The former are inverted structures of the latter. In the Scaffolds \( a \) and \( b \), the depth of micro-holes and the height of pillar-like structure are about 160 nm and 200 nm, respectively. These values of \( Ra \) and \( Rku \) in Scaffold \( a \) are almost same as that of Scaffold \( b \). There is major difference in the value of \( Rsk \) between Scaffold \( a \) and \( b \). Table 1 shows a list of surfaces prepared as a cell scaffold in this present study; the value of \( Rsk \) is ranged from -0.55 to 0.81, \( Rku \) 2.7 to 3.3, and \( Ra \) 13 to 40 nm. These micro-structured scaffolds are easily fabricated as compared to that semiconductor process.

### 3.2 Effects of surface geometries on cell adhesion

This present study cultured Rat phenochromocytoma (PC12) on the above described PDMS scaffolds and a substrate with self-assembled particles. PC12 cells were provided from RIKEN BioResource Center. After UV sterilization for 24 hours, the fabricated PDMS scaffolds were immersed in a dish with standard culture solutions, which consisted of Dulbecco’s modified Eagle medium, fetal bovine serum (10 wt%), horse serum (10 wt%), and penicillin-streptomycin (10 mL/L). PC12 cells were seeded at 5000 cells/cm\(^2\), and then incubated at 310 K in a 100% humidity atmosphere consisting of 95% air and 5% \( CO_2 \). The PDMS scaffold surfaces were observed by phase-contrast microscopy (IX-71, Olympus Co. Ltd.) at 24 h after cell seeding. To evaluate spatial selectivity of cell adhesion, the authors define the following evaluation values; selective adhesion ratio \( \alpha \) as expressed in Equation (4),

\[
\alpha = \frac{N_m}{(N_m + N_f)}
\]

where \( N_m \) and \( N_f \) are the number of adhered cells counted on the surface of micro-structured regions and the surface of flat regions, respectively.

Figure 6 shows phase microscope images of the micro-structured PDMS scaffolds at 24 hours after cell seeding. The cultured cells were adhered on all the scaffolds, and they were scattered on the scaffold surface. A selective cell adhesion to micro-structured regions, however, was also found on some scaffolds. The number of cells adhered on the micro-structured regions of Scaffold \( a \) (\( Rsk = -0.6 \)) is larger than that of Scaffold \( b \) (\( Rsk = 0.8 \)). Furthermore, the scaffold of self-assembled particles (\( Rsk = -1.5 \)) has higher selective adhesion of cells as shown in Fig. 6 (c) (Takeda, et al., 2012; Takeda, et al., 2015). There was obvious difference in selectivity of cell adhesion between scaffolds, although it markedly appeared on the self-assembled particles. It is found that the difference is attributed to surface geometries. Figures 7 shows the graphs of selective adhesion ratio, \( \alpha \), which is plotted against the value of \( Rsk \) and \( Ra \) of the scaffold surfaces. The numbers of adhered cells were counted from the phase microscope images to calculate the selective adhesion ratio shown in Eq. (4). One can find that a downward of trend of the selective adhesion ratio \( \alpha \) with \( Rsk \), although there is poor correlation between \( \alpha \) and \( Ra \). The value of \( Rku \), however, did not affect cell adhesion as well as that of \( Ra \). For example, Scaffold \( a \) (\( Rsk = -0.6 \)) and \( b \) (\( Rsk = 0.8 \)) have almost same value of \( Ra \) (40 - 50 nm), but the value of \( \alpha \) on Scaffold \( a \) is larger than that of Scaffold \( b \) (Scaffold \( a \): 73% and Scaffold \( b \): 56%). These experimental results summarizes as follows. The cells were preferentially adhered on the micro-structured scaffold with micro-holes array, which has lower \( Rsk \) as shown in Fig. 6 (a). The reason why the scaffold of self-assembled particles have the highest value of \( \alpha \) (> 80%) is attributed to a function of micro-gaps as a flow channel in addition to surface geometries (Takeda, et al., 2015).

These results indicate that the cells are tends to adhere on a top surface with gently mounds as compared to that of sharp peaks. These experimental results are considered as follows. Cell generally adheres on a scaffold surface via cell-adhesive proteins by its filopodiums. Therefore, in the case of micro-structured scaffold with aligned segments, the top surface of segment have requires enough area to contact with cell filopodium. The tip area of filopodium is estimated to be about 0.25 \( \mu m^2 \) from the area of focal adhesion of cell (Zaidel-Bar, et al., 2004). The area of focal adhesion gradually increases to about 1 \( \mu m^2 \) with cell growth (Aldo, et al., 2010). In consideration of these previous researches, the segment of micro-structured scaffold is expected to have larger surface area than 0.25 \( \mu m^2 \) in cell adhesion. The pitches of micro-structure (segment) are same for all the scaffolds in this present study. Therefore, the small (negative) \( Rsk \) (<0) with larger segment area enhances cell adhesion, and also induces selective cell adhesion on a line-and-space pattern of micro-structured scaffold.
4. Conclusions

This study can be concluded as follows. The number of the adhered PC12 cells on surface tends to their skewness while surface roughness and kurtosis are not affected. Negative skewness leads to improve cell adhesion is found. $R_{sk}$ of surface is important for cell adhesion.

![Diagram of surface geometries](image)

**Figure 1** Schematic illustration of surface geometries.

![Fabrication procedure](image)

**Figure 2** Fabrication procedure of structured substrate for cell scaffold. (a) Self-assembly of micro-particles. (b) Dry etching to Si wafer. (c) Molding of PDMS.
only the hydrophilic regions. (b) Reactive ion etching using self-assembled particles mask to Si wafer. The Si wafer is locally etched off to form an array of micro-pillar. The micro-structured Si is utilized for a master mold of cell scaffold. (c) Molding procedure of PDMS. A pre-polymer of PDMS is casted into the etched Si surface and then thermally cured. This micro-structured PDMS is used as a cell culture scaffold or a second master mold for an inverted structure.

Figure 3 SEM images of micro-structured SiO$_2$ particles. Regularly patterned particles are found. The line width and spacing of micro-structured particles were about 30-40 μm and 70-60 μm, respectively.

Figure 4 SEM image of a Si wafer after dry etching with particle mask. Regularly patterned taper shape structures are found.
Figure 5 AFM images and profile curves of micro-structured PDMS. Scaffolds a, c, and e have hole-like structures. Scaffolds b, d, and f have pillar-like structures.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Ra</th>
<th>Rs</th>
<th>Rku</th>
<th>Configuration</th>
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<tbody>
<tr>
<td>a</td>
<td>-0.6</td>
<td>2.7</td>
<td>40</td>
<td>Hole</td>
</tr>
<tr>
<td>b</td>
<td>0.8</td>
<td>2.7</td>
<td>46</td>
<td>Pillar</td>
</tr>
<tr>
<td>c</td>
<td>-0.2</td>
<td>3.1</td>
<td>22</td>
<td>Hole</td>
</tr>
<tr>
<td>d</td>
<td>0.2</td>
<td>3.3</td>
<td>19</td>
<td>Pillar</td>
</tr>
<tr>
<td>e</td>
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<td>2.8</td>
<td>30</td>
<td>Hole</td>
</tr>
<tr>
<td>f</td>
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<td>3.0</td>
<td>13</td>
<td>Pillar</td>
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<tr>
<td>g</td>
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<td>2.7</td>
<td>147</td>
<td>Particles</td>
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</tbody>
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Figure 6 Phase contrast images of cultured PC12 cells on structured PDMS and the effect of $R_{sk}$ and $Ra$ on cell adhesion. (a) - (c) Phase contrast images of adhered cells. These images are typical examples. The cells selectively adhere on structured region in the case of (a) and (c).

Figure 7 Effects of $R_{sk}$ and $Ra$ on cell adhesion. Error bars indicate standard deviation.

References


