

## Quantitative Analysis of Growth of Cells on Physicochemically Modified Surfaces

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In this study, we describe the most expected behavior of cells on the modified surface and the correlation between the modified substrates and the response of cells. The physicochemical characteristics of substrates played an essential role in the adhesion and proliferation of cells. Glass and polymer substrates were modified using air plasma oxidation, and the surfaces were coated with self-assembled monolayer molecules of silanes. The PDMS substrates embedded with parallel micropatterns were used for evaluation of the effect of topologically modified substrate on cellular behaviour. BALB/3T3 fibroblast cells were cultured on different surfaces with distinct wettability and topology, and the growth rates and morphological change of cells were analyzed. Finally, we found the optimum conditions for the adhesion and proliferation of cells on the modified surface. This study will provide insight into the cell-surface interaction and contribute to tissue engineering applications.

**Key Words :** Cell-surface interaction, Surface modification, Contact angle, Growth curve

### Introduction

In the development of biomedical devices, biosensors, and artificial organs, it is crucial to understand the relationship between biomaterials and biological tissue at the cellular level.<sup>1</sup> The three most important factors responsible to the cell fate are cell-cell interaction, cell-matrix interaction, and cell-soluble factor interaction. The cell-matrix interaction plays a fundamental role in the regulation of survival, proliferation, migration, and differentiation of adherent cells on the substrates. Surface properties such as wettability, roughness, surface charge, and chemical functional groups are most important for the cell adhesion.<sup>1</sup> The ability to support adhesion and proliferation of cells is a prerequisite for any material to serve as a scaffold for tissue engineering. The interactions between receptors in the cell membrane and specific ligands on the supporting substrate play a primary role in cellular behavior. In natural environments, such interactions could be acquired through the adhesion peptide sequences of extracellular matrix (ECM) proteins, including RGD, YIGSR, *etc.*<sup>2-4</sup> In the development of biomaterials, adhesive molecules on the surface, which are either chemically or physically modified, are mainly responsible for the adhesion of cells. In earlier reports, different surfaces with several modifications techniques have been reported individually, which consist of different wettabilities, functional groups, surface densities, roughness and rigidity.<sup>5-9</sup> The relationship between roughness and wettability has been reported by Ponsonnet *et al.* and they found that low surface energy components over a smooth surface are more favorable to the proliferation of fibroblasts.<sup>10</sup> Various properties have been reported for the cell-matrix interaction, and moderate wettability of a water contact angle of 40-70° in polymeric materials allows for better adhesion of cells.<sup>5-7</sup>

The 3-dimensional morphology of the substrate is also an

important factor that affects the reactivity of adherent cells. Surface topography is an important environmental cue for controlling cellular responses from initial attachment and migration to proliferation, differentiation, gene expression, and production of new tissue.<sup>11-13</sup> Various studies demonstrated that cells are sensitive to the gross morphology of a substrate, and substrates have been examined with their roughness to interpret the cell-surface interaction. Aligning the cells along the microstructure elongates them because of the contact guidance of cells.<sup>11,12</sup>

The surface of biomaterials for implants is exposed to numerous proteins present in blood, interstitial fluid, and damaged ECM, which forms a complex layer of adsorbed proteins at the surface of the materials.<sup>14,15</sup> The behaviour of cells in a serum or protein-containing medium is affected by proteins adsorbed at the surface of the materials.<sup>15</sup> The adhesion proteins that are present in blood, serum, and the ECM, such as fibronectin, vitronectin, collagen, and laminin, contain specific amino acid sequences that bind to the integrin receptors of the surface of cells, and affect the behaviour of cells and gene expression.<sup>14,15</sup> Integrin ligation by the cell is followed by receptor aggregation and accumulation of extracellular proteins, which forms the focal adhesion that integrates the ECM to the cell.<sup>16,17</sup> The hydrophilic surface facilitates the adhesion protein with adsorption and the focal adhesion that regulates the extracellular protein formation. All of the adherent cells over the modified substrate respond to the cell viability, growth rate, and proliferation.<sup>18-20</sup>

In this study, we describe the correlation between the cell's response and the surface properties. We used different model surfaces with different degrees of wettability and microstructures to examine their influence on the adhesion and proliferation of cells. Polystyrene culture plates, PDMS thin films with micropatterns, and glass surfaces with a self-assembled monolayer of methyl-, amine-, and thiol-functional

groups have been used for studying the correlation between cell-surface interaction. Cell adhesion and growth were assessed by counting the cells from images taken over various time intervals and a growth curve was obtained. We observed the wettability and topology of the surface showing an influence on the growth and proliferation of cells. This study could be helpful in understanding how the surface properties of materials influence the cellular response and in engineering implantable materials and artificial tissues.

## Experimental

**Preparation of the Substrate.** Polystyrene culture plates (6-well plates, CellStar<sup>®</sup>, Greiner Bio-One GmbH, Germany) were used as a control for the adhesion and proliferation of fibroblast cells. Glass coverslips ( $18 \times 18 \text{ mm}^2$ ) were received from Marienfeld (Germany). They were cleaned by immersion in 2% aqueous Micro-90<sup>TM</sup> cleaning solution (Cole Parmer Instrument Co., USA) at room temperature for 24 h and sonicated in cleaning solution for 5 min. The cleaned glass coverslips were repeatedly rinsed in deionised (DI) water (10 times) and dried before use. The polymer substrate embedded with parallel micropatterns was fabricated in PDMS (Dow Corning, Sylgard 184, USA) using rapid prototyping and soft lithography techniques.<sup>21,22</sup> The master for the micropatterns was fabricated by patterning of photoresists. Briefly, photoresist, 2-5  $\mu\text{m}$  thick, was obtained by spinning SU-8 5 (Microchem, MA, USA) negative photoresist on a 3" silicon wafer at 2000-4000 rpm for 60 s. A mask (Nano Fab Center, Korea) was used to pattern the SU-8 5 photoresist. A thin layer of PDMS was prepared by mixing prepolymer and a curing agent in a 10:1 ratio and then adding it to a polystyrene petri dish containing the master. The mixture was degassed in a vacuum desiccator and then heated in an oven at 70 °C for 4 h. PDMS substrate was cut into a square shape of  $18 \times 18 \text{ mm}^2$  (~0.5 mm thick), sterilized in 70% ethanol, and kept in a clean place for further use.

**Modification of Surfaces with Air Plasma.** The surface of coverslips was activated with reactive oxygen plasma (Harrick Scientific, PDC-002 Plasma Cleaner; 30 W, ~0.4 mmHg) for 30 s, and was used for cell seeding and proliferation assessment. The surface of the PDMS thin films was activated with reactive oxygen plasma for 30 s and exposed to the air for a different time interval in order to obtain different contact angles over the PDMS substrate, and then was used for cell culture.

**Modification of Surfaces with Self-Assembled Monolayer (SAM).** The modification of surfaces was performed as following: 3-(Aminopropyl)trimethoxysilane (APTMS, Fluka, USA), 3-mercaptoptrimethoxysilane (MPTMS, Aldrich, USA), and octadecyltrichlorosilane (OTS, Aldrich, USA) were used as received. In order to prepare the surface with amine groups, coverslips ( $18 \times 18 \text{ mm}^2$ ) cleaned with oxygen plasma were immersed in an ethanolic 1% APTMS solution for 30 min, washed with ethanol, and then dried under the blowing of N<sub>2</sub> gas. Thiol-modified glass coverslips were

prepared by immersing clean cover slips in a methanolic 1% MPTMS solution for 30 min, washed with methanol, and then dried under the blowing of N<sub>2</sub> gas. 0.01% of OTS solution in hexane was prepared and cleaned coverslips were dipped in the solution for 1 min, washed with isopropyl alcohol, and then dried under the blowing of N<sub>2</sub> gas. Finally, coverslips treated with SAM molecules were kept at 105 °C for 10 min to complete the formation of chemical bonds between molecules and substrates.

**Measurement of Water Contact Angles.** For an evaluation of surface wettability, the water contact angles of the modified substrates were measured at room temperature using a contact angle goniometer (Model G-1 type, Erma Inc., Japan). A small droplet of water was placed on the air-side surface of the substrate and the contact angle was measured after 30 s. More than 10 measurements were carried out for each sample and the values were averaged.

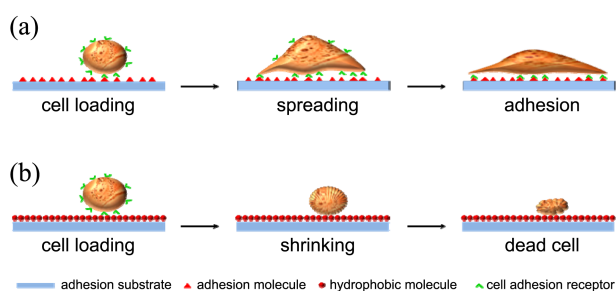
**Culture of Fibroblast Cell.** BALB/3T3 fibroblasts (Korean Collection for Type Cultures) were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma, USA) supplemented with 10% Fetal bovine serum (FBS) (Invitrogen, USA) and 1% penicillin and streptomycin (Sigma, USA). For a routine culture and in all experiments, the cells were maintained at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere. For the initial cell adhesion experiment, cells with a density of  $1 \times 10^4$  cells/mL were loaded on each substrate, the dead cells were removed by suction after 1 h, and the substrates were washed with HBSS, and then fresh growth media were added.

Cells were fixed in 4% paraformaldehyde (Aldrich) for 15 min at 37 °C followed by a washing with PBS. Subsequently, cells were permeabilized with 0.5% Triton X-100 (Aldrich) in PBS for 5 min at 4 °C. The cells were then blocked with 1% BSA (Sigma) in PBS for 5 min at 37 °C. Cells were further exposed to rhodamine-conjugated phalloidin (1:100 in 1% BSA/PBS, Molecular Probes) to visualize the F-actin (actin filaments).

**Microscopy and Cell Imaging.** Live cell images were captured by inverted microscope, Olympus IX71 equipped with DP71 CCD camera. Images were collected in a regular interval, and the numbers of cells were counted by image analyses software (Image Pro-Plus<sup>®</sup> version 7.0, Media Cybernetics, USA). All images were obtained as bright-field images and were standardized for further analysis. Growth curves were plotted for the fibroblast cell cultures over different modified substrates.

## Results and Discussion

In this study, we tried to understand the relationship between surface properties of substrates and the rate of proliferation of cells. A schematic illustration of the attachment and spreading of cells on the different substrates is shown in Figure 1. Cells were attached, spread, and proliferated on the preferred surface, while they were detached and washed away from the non-preferred substrate. Therefore, surfaces were prepared with different modification methods, and the



**Figure 1.** Schematic illustration for the loading, spreading, and adhesion of cells on modified surfaces. (a) The loaded cells are spread out and adhere on the adhesion substrate, (b) cells are shrunk and washed away from non-favourable substrate.

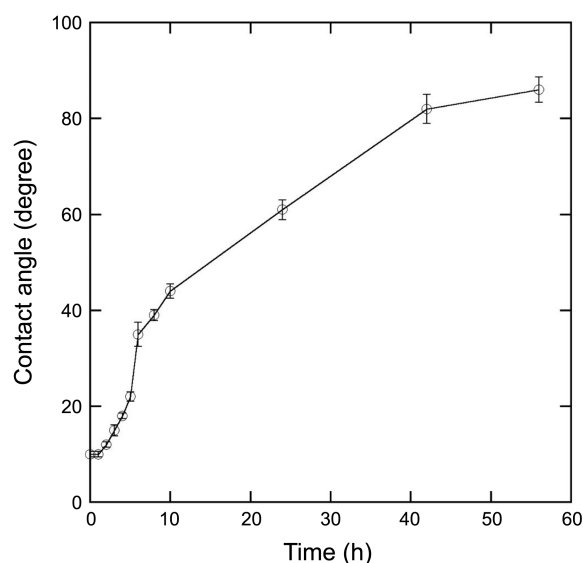
growth curves of fibroblast cells on the different substrates were obtained in order to evaluate the relationship between modified substrates and cell growth.

**Water Contact Angles of the Substrate.** Water contact angles indicate the wettability of the substrate. Static water contact angles were measured by the sessile drop method on the modified surfaces and the results were summarized in Table 1. The contact angle of the polystyrene tissue culture dish, which was used as a reference surface, was  $62^\circ$ , and the contact angle of the glass coverslips treated with air plasma was  $21^\circ$ , the values are in agreement with the reported values.<sup>23</sup> Usual PDMS surface showed super hydrophobic properties with a contact angle of  $110^\circ$  because the surface was covered with nonpolar methyl groups.<sup>27</sup> By the treatment of air plasma on the PDMS surface for 30 s, polar silanol groups were developed on the surface, and the water contact angle decreased to  $\sim 10^\circ$  as reported by others.<sup>27</sup> Because the hydroxyl groups form a strong intermolecular bond between surfaces and water, they make the surface highly hydrophilic.<sup>27</sup> However, the hydrophobic characteristics of plasma-treated PDMS were recovered while they were placed in the air because hydrophilic moieties moved inside the matrix in order to reduce surface energy.<sup>27</sup> As a result, the contact angles increased again as a function of time after exposure to the air as shown in Figure 2. After 2 days, the contact angles of PDMS surfaces increased to  $85^\circ$ , a value very similar to that of the non-treated surface.<sup>27,28</sup>

Contact angles of coverslips modified with various SAM

**Table 1.** Static contact angles of water of several substrates

Substrate	Contact angle ( $^\circ$ )		Reference
	Measured	Reported	
polystyrene culture plate	$62 \pm 4$	65	23
glass coverslip			
plasma treated	$21 \pm 2$	22	23
APTMS treated	$57 \pm 2$	59	24
MPTMS treated	$64 \pm 4$	67	24
OTS treated	$110 \pm 3$	113	25
PDMS substrate			
non treated	$110 \pm 4$	114	26
plasma treated	$< 10$	$< 10$	27

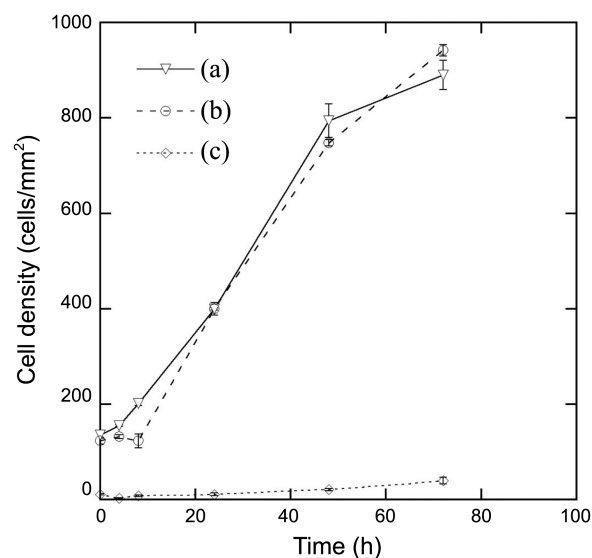


**Figure 2.** Change of static contact angles of plasma oxidized PDMS surface as a function of time allowed in the air.

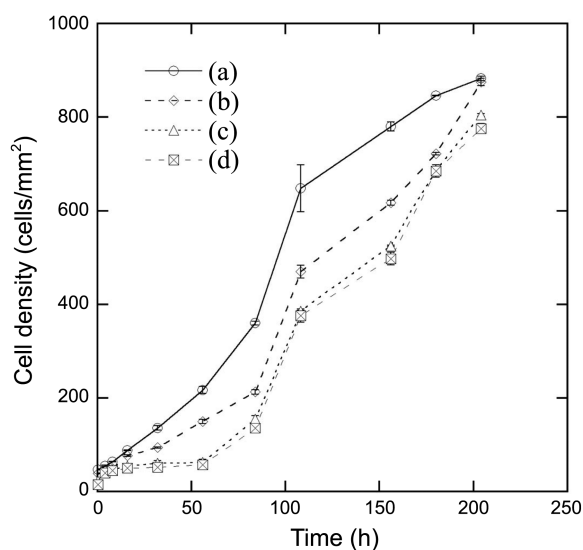
molecules of MPTMS, APTMS, and OTS were also summarized in Table 1. Nonpolar  $-\text{CH}_3$  groups in OTS-treated surfaces showed very hydrophobic properties with a contact angle of  $110^\circ$ , while polar  $-\text{SH}$  and  $-\text{NH}_2$  groups showed moderate contact angles of  $64^\circ$  and  $57^\circ$ , respectively.

Contact angles of plasma-treated PDMS substrates embedded with micropatterns showed low contact angles of  $\sim 10^\circ$ , implying the surfaces were hydrophilic and the contact angle is independent of both the spacing and depth of micropatterns.

**Adhesion and Proliferation of Balb/3T3 Fibroblast Cells on the Control Substrates.** Adhesion and proliferation of cells on the control substrates were analyzed with the live cell imaging system using Image-pro software. Cells



**Figure 3.** Cell growth curves of BALB/3T3 fibroblast cells on three different surfaces. (a) glass substrate, (b) tissue culture grade polystyrene substrate, and (c) non-treated PDMS substrate.

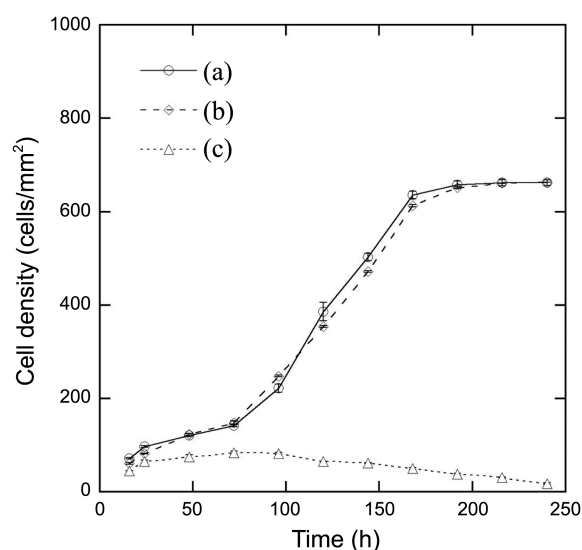


**Figure 4.** Cell growth curves of BALB/3T3 fibroblast cells on the plasma-oxidized PDMS substrate (a), PDMS allowed in the air for 0 min, (b) 20 min, (c) 2 h, and (d) 4 h.

were loaded on polystyrene culture plates, glass, and non-treated PDMS substrates with the same cell densities ( $\sim 100$  cells/mm<sup>2</sup>). The growth curves were plotted and the proliferation rates were measured for the control surfaces as shown in Figure 3. Cells were attached, spread, and proliferated on the polystyrene culture plate and glass substrate. Growth rates of cells on both substrates were almost the same. However, most of the cells were detached and washed away from the non-treated PDMS substrate and a few cells were found in the case of the PDMS substrate. Both the glass substrate and polystyrene culture plate gave enough hydrophilic surface to attach, spread, and proliferate, while the PDMS substrate gave a hydrophobic surface that cells could not attach to the surface.

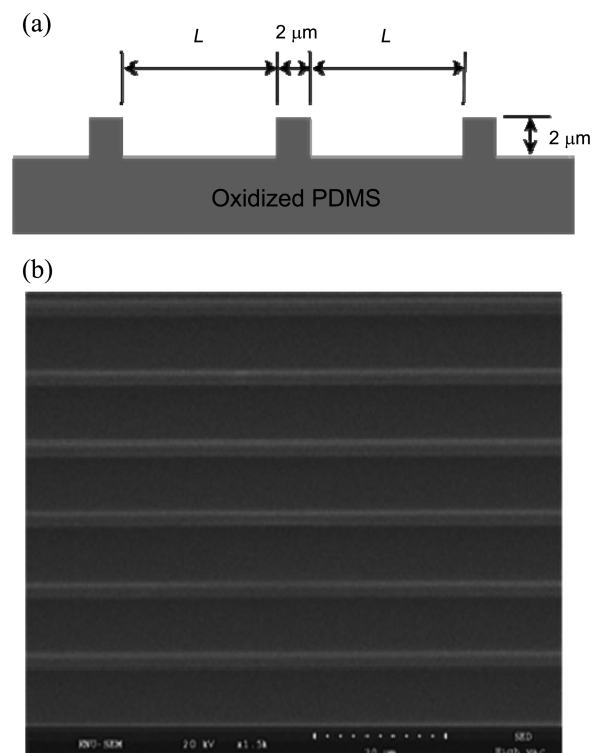
The growth curves of BALB/3T3 fibroblast cells on the different PDMS surfaces are shown in Figure 4. The non-treated PDMS substrate showed hydrophobic properties with a contact angle of  $110^\circ$ , and poor cell adhesion was observed on that surface. Many adherent cells were observed on the plasma-treated PDMS surface (Figure 4(a)). Because the hydrophobic characteristics of plasma-treated PDMS were recovered while they were placed in the air, the growth rate of cells on the substrate exposed to a longer time in the air was slower (Figure 4(b)-(d)).

Growth curves of cells on the SAM-treated glass substrate are shown in Figure 5. Different functional groups provide a specific charge and binding on the surface. APTMS-modified and MPTMS-modified substrates showed very similar water contact angles to that of the control surface (polystyrene culture plate) and showed very similar growth curves. APTMS- and MPTMS-modified surfaces created a favourable environment for cell adhesion and proliferation and  $-\text{NH}_2$  groups on the substrate showed better adhesion and spreading. Due to the very hydrophobic nature of the OTS-treated surface, the adhesion of cells was avoided and cells could not grow on the surface.

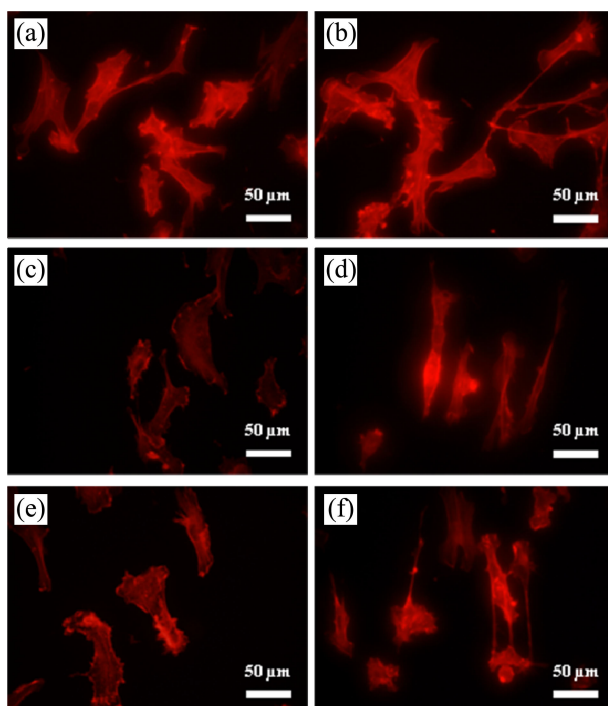


**Figure 5.** Cell growth curves of BALB/3T3 fibroblast cells on the glass substrates coated with (a) APTMS, (b) MPTMS, and (c) OTS.

**Adhesion and Proliferation of Balb/3T3 Fibroblast Cells on the Micropatterned Substrate.** The approximate dimension of cells is around  $30\text{--}50$   $\mu\text{m}$ . Cells might be polarized on the micropatterned PDMS substrates. A schematic illustration of micropatterned PDMS substrates with repeating plateau patterns is shown in Figure 6(a). The width



**Figure 6.** (a) Schematic illustration of micropatterned PDMS substrates with repeating plateau patterns. The width of the plateau is  $2$   $\mu\text{m}$ , the height of the plateau is  $2$   $\mu\text{m}$ , and the spacing ( $L$ ) of plateaus are  $2\text{--}20$   $\mu\text{m}$ . (b) SEM image of plateau patterns with  $2$   $\mu\text{m}$  wide,  $2$   $\mu\text{m}$  high, and  $7$   $\mu\text{m}$  spacing.

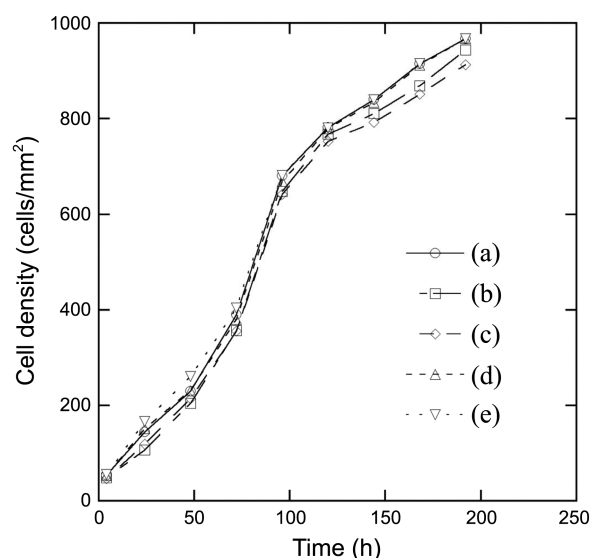


**Figure 7.** Fluorescent microscopic images of cells cultured on the different substrates. (a) glass substrate, (b) plasma-oxidized PDMS, plasma-oxidized PDMS substrates with different plateaus spacing with (c) 2  $\mu\text{m}$ , (d) 3  $\mu\text{m}$ , (e) 7  $\mu\text{m}$ , and (f) 15  $\mu\text{m}$ . Cells were stained with rhodamine-conjugated phalloidin after culture.

of the plateau is 2  $\mu\text{m}$ , the height of the plateau is 2  $\mu\text{m}$ , and the spacing of plateaus varies from 2 to 20  $\mu\text{m}$ . SEM image of PDMS substrate embedded with plateau patterns with 2  $\mu\text{m}$  wide, 2  $\mu\text{m}$  high, and 7  $\mu\text{m}$  spacing is shown in Figure 6(b). Figure 7 shows fluorescent microscopic images of cells cultured on the various substrates. Cells cultured on the flat substrates of glass and oxidized PDMS are spread out on the substrate (Figure 7(a) and (b)). Cells cultured on the plateaus with 2–7  $\mu\text{m}$  spacing were found to be adherent on the plateaus and were elongated and oriented along the direction of plateaus as shown in Figure 7(c)–(e). Microtubule and filamentous intracellular structures play a direct role in controlling cytoskeleton and morphological aspects of contact guidance. Alterations in the shape of cells are associated with a change in the cytoskeleton that generates the mechanical force important for cell and tissue functions.<sup>29,30</sup> Cells on the plateaus with spacing greater than 15  $\mu\text{m}$  were similar to cells cultured on the flat substrates. In both cases, the cells were grown in the wide groove regions (Figure 7(f)).

Growth curves of cells on the micropatterned substrates are shown in Figure 8. Growth rates of cells on the substrate with plateaus were affected by the spacing of plateaus. The growth rates of cells on the plateaus with 2–3  $\mu\text{m}$  spacings are somewhat lower than that of the control, because cells were polarized on the substrate. The cytoskeleton controls the mechanotransduction which influences the vital functions of cells like growth, proliferation, migration, and gene expression. Mechanotransduction responds to the conformational change in the cytoskeleton and passes chemical

and mechanical signals to the nucleus about the extracellular environment.<sup>31,32</sup> Environmental cues, topography, soluble growth factors, and integrin-mediated adhesion induce morphological alteration and cytoskeletal protein organization in all types of cells. Attachment of cells to the ECM or other cells has long been implicated in cell cycle regulation. During cell division, the cells undergo extensive cell shape changes to detach from and reattach to the ECM. While cell-matrix adhesions have been reported to decrease during mitosis, in adherent cell types, cell rounding upon entry into mitosis is accompanied by a reduction in the focal contacts and an increase in cortical rigidity.<sup>33</sup> After cytokinesis, cells reattach to their substratum and re-establish cytoskeleton networks. Changes in gross morphology and contact guidance over microstructured surfaces may result in cell cycle arrest or may slow down the cell cycle.<sup>34</sup> In our work, the growth of cells was reduced on the micropatterned surfaces with narrow spacings compared to the smooth surfaces. Cells have been found increasingly able to descend into the grooves and form focal adhesions on the grooves as the ridge width increased and the groove depth decreased.<sup>35</sup> Cell and cytoskeletal alignment has generally been found to be more pronounced on patterns with ridge widths between 2 and 3  $\mu\text{m}$  than on grooves and ridges with larger lateral dimensions. den Braber *et al.* showed that the cell proliferation was not affected by either the presence of the microgrooves or the dimension of the grooves. However, they reported that smaller ridges showed variation in growth rate initially and the proliferation of cells in microgrooves appeared to be dependent on the types of cells.<sup>36</sup> In most studies, substrata with grooves of 0.45  $\mu\text{m}$  to 1.0  $\mu\text{m}$  deep were used. In the present study, we have increased the plateau depths at which cells will respond significantly to topography. Wang *et al.* reported that the cell proliferation on the substrate with



**Figure 8.** Cell growth curves of BALB/3T3 fibroblast cells on the oxidized PDMS substrates with different plateau spacing. (a) plasma-oxidized PDMS, and spacings are (b) 2  $\mu\text{m}$ , (c) 3  $\mu\text{m}$ , (d) 7  $\mu\text{m}$ , and (e) 15  $\mu\text{m}$ .

various groove/ridge widths (12/12, 18/18 and 24/24  $\mu\text{m}$ ) and fixed depth of 2  $\mu\text{m}$  was not different from that on the smooth surfaces, however, a similar influence on cellular alignment was found. This means that the behavior of cells can already be influenced by very shallow grooves.<sup>37</sup>

It could be speculated that micropatterns reduced adhesion, and it impacts on integrin-related signaling through the reduction of sites for adhesion and cytoskeletal anchorage. This further reduced tension applied to the cell from a well-organized extracellular matrix *via* the cytoskeleton to the nucleus, effectively shutting the cells down to transcription, leading to a reduced growth rate. The growth rates of cells on the plateaus with 7-15  $\mu\text{m}$  spacings were almost same with the control because cells were cultured in the wide microgroove regions, and they were less polarized. Our research supports the findings that physicochemical parameters such as wettability and surface free energy influence cell growth whereas the orientation and shape of cell depends upon the spacing of plateaus.

### Conclusion

In this work, a correlation between the wettability and morphology of the surface and the proliferation of cells was investigated. Water contact angles in the range of 10-85° were obtained with a PDMS substrate allowed in the air after plasma exposure, which led to a progressive reduction in the level of cell adhesion and the rate of growth. Following phenomena represents the favorable wettability for cell adhesion and growth on biomaterial surface. Higher growth rate obtained in moderate wettability substrates. It was demonstrated that there might be a wettability threshold (40-70°) for cell proliferation and it becomes difficult to proliferate on the surfaces with a higher contact angle. As a result, the adhesion and proliferation of cells may be reduced. With chemical modification by silanes of APTMS, MPTMS and OTS, adhesion and growth of cells were influenced by surface chemistry and surface free energy. This might suggest that adsorption of proteins from the culture medium was influenced by the surface property and by the initial interaction of cells with these proteins that supported the adhesion and proliferation of cells. The adhesion of cells was better on a hydrophilic surfaces, while the proliferation of cells was significantly higher on a moderate-wettability surface. Cells cultured on the plateaus with spacings of 2-7  $\mu\text{m}$  were found to be adherent on the plateaus and were elongated and oriented along the direction of plateaus. The growth rates of cells on the substrates with plateaus were affected by the spacings of plateaus. The results described in this work are significant for the assessment and design of new surfaces for biological applications and it is suggested that surface property be taken into account in the designing of new biomaterials, especially orthopaedic implants, and tissue engineering applications.

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