

Effects of Biomimetic Micropatterned Surfaces on the Adhesion and Morphology of Cervical Cancer Cells

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Cite This: *ACS Omega* 2022, 7, 19913–19919

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ABSTRACT: It has been demonstrated that micropatterned surfaces have an important influence on modulating cellular behavior. In recent years, with the rapid development of microfabrication techniques and in-depth study of nature, an increasing number of patterned structures imitating natural organisms have been successfully fabricated and widely evaluated. However, there are only a few reports about biomimetic patterned microstructures in biologically related fields. In our work, micropatterned polydimethylsiloxane (PDMS) was fabricated by mimicking the surface microstructures of natural *Trifolium* and *Parthenocissus tricuspidata* leaves using the template duplication method. The interactions between the two types of biomimetic micro-PDMS surfaces and two kinds of human cervical cancer cells (HeLa and SiHa) were investigated. HeLa and SiHa cells cultured on the two micropatterned PDMS samples exhibited more stretchable morphology, higher diffusion, and a much lower nuclear/cytoplasmic ratio than those cultured on flat PDMS surfaces, indicating a higher adhesion area of the cells. Both of the micro-PDMS substrates were found to induce significantly different morphological changes between HeLa and SiHa cells. This suggests that the micropatterned structure affects cell adhesion and morphology correlated with their surface geometric structure and roughness. The results reveal that biomimetic micropatterned surfaces from natural leaves significantly regulate the morphology and adhesion behavior of cervical cancer cells and are believed to be the new platforms for investigating the interaction between cells and substrates.



1. INTRODUCTION

Textured substrates play a key role in regulating cell behaviors, such as cell adhesion and morphology,^{1–3} cell migration,^{4–6} and differentiation.^{7–9} Moreover, cells can sense and respond to the microtopography of the substrate, which largely depends on the physical and chemical properties, geometries and feature dimensions of the substrate itself, and the cell types.^{10,11}

Currently, an increasing number of artificial patterned structures at the microscale have been prepared and used as biointerfaces for *in vitro* cell culture benefiting from various microfabrication strategies.¹² One of the strategies that has recently received much attention is the template method,¹³ in which a material with a special structure was used as the template to redefine its structural pattern onto the other product. The template method is deemed a simple yet effective way to fabricate bionic surfaces with desired patterned structures. For example, Wang and Lu developed a template method where an anodized aluminum oxide (AAO) membrane was used as a template to successfully fabricate ordered patterned structures for a periodontal ligament fibroblast (PDL) culture.¹⁴ Chong et al. used a polyallyldiglycol carbonate (PADC) film with micron-scale spherical pores as a template to obtain a micropillar substrate. HeLa cells cultured on micropillar substrates had significantly larger cell

spreading areas and higher cell numbers.¹⁵ However, the process to prepare templates is usually costly and time-consuming and requires specific equipment. Therefore, new methods intending to reduce production cost and simplify the preparation process are presently under active investigation.

After millions of years of natural selection and evolution, plant leaves generally have nearly perfect structures on the upper epidermises to adjust themselves in different environments.¹⁶ The epidermal microstructures of leaves have also been the theme of many researchers. For instance, lotus leaves^{17,18} with protrusions and nanorod microstructures have been successfully applied for building bionic self-cleaning surfaces because of their natural superhydrophobicity as well as rose petals.^{19–21} In addition, some special features of multiple leaves, such as corn, lotus, *Ilex chinensis* Sims, and *Photinia serrulata*, were also applied to design photocell antireflection (AR) structures.²² Accordingly, leaves can be used as templates

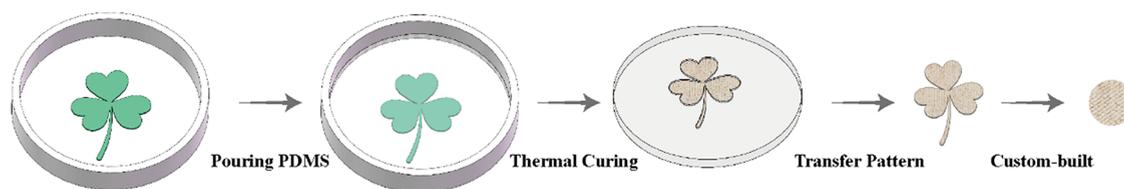
Received: March 21, 2022

Accepted: May 17, 2022

Published: May 27, 2022



Scheme 1. Schematic Illustration of the Preparation Process of the Trifolium-Templated Micropatterned PDMS Sample



to fabricate patterned structures to be biointerfaces for cell culture. Various leaves can be easily obtained in nature, which will avoid a complex template preparation process, and moreover, biomimetic patterned surfaces are capable of inheriting the physicochemical properties of natural biological surfaces, which may be beneficial for cell culture on biointerfaces.

High-risk human papilloma viruses (HPVs) such as SiHa (HPV-16) and HeLa (HPV-18) have been attributed to be the major risk factors for cervical cancers. In this study, for investigating the growth of HeLa and SiHa cervical cancer cells, two natural *Trifolium* and *P. tricuspidata* leaves with the excellent properties of hydrophobicity and surface morphologies were used as templates to develop island-like and stripe-like biomimetic patterns on polydimethylsiloxane (PDMS) surfaces. Owing to the outstanding biocompatibility of PDMS, many efforts have been made to perform biomedical works based on PDMS, for example, for microfluid chips²³ and cell behavior.^{24,25} Interestingly, the two biomimetic PDMS patterns have a strong effect on cervical cancer cellular adhesion behavior and morphology. HeLa and SiHa cells cultured on the two micropatterned PDMS substrates showed larger stretchable morphology, higher diffusivity, and a lower nuclear/cytoplasmic ratio than those cultured on a flat PDMS surface, indicating that the cells had higher adhesion. This reveals that the micropattern structures affect the cell morphology, which is related to its surface geometry and roughness. The results show that the bionic micropatterned surface of natural leaves can significantly regulate the morphology and adhesion behavior of cervical cancer cells, which can be considered to be a new platform for investigating the interaction between cells and substrates.

2. MATERIALS AND METHODS

2.1. Materials. Sylgard-184 silicone elastomer (Dow Corning Corporation), *n*-hexane (97%), and trichloromethane (99%) were acquired from Tianjin Chemical Reagents Corp. Two kinds of plant leaves, *P. tricuspidata* and *Trifolium*, were taken directly as templates for biomimetic surface duplication. An RPMI-1640 medium (Gibco, USA), fetal bovine serum (Sera Pro, USA), penicillin–streptomycin solution (Sigma-Aldrich, USA), phosphate saline buffer solution (PBS) at pH = 7.4 (Solarbio, Beijing), trypsin 0.25% solution (Hy-Clone, USA), formaldehyde solution (3.7%, Zhongqin, Shanghai), 2.5% glutaraldehyde solution (Kelong, Chengdu), fluorescent anti-decay sealants (Solarbio, Beijing), Triton X-100 (Amresco, USA), CytoPainter Phalloidin-iFluor 488 Reagent (Abcam, USA), and DAPI (4',6-diamidino-2-phenylindole, Sigma-Aldrich, USA) were used as received.

2.2. Fabrication of Biomimetic Micro-PDMS Pattered Surfaces. As shown in previous studies,^{26–28} microstructured PDMS has been developed by a simple template duplication method (Scheme 1). First, *Trifolium* and *P. tricuspidata* leaves were attached to a glass dish. Second, the PDMS precursor was

prepared by mixing a Sylgard-184 elastomer base with a curing agent in a 10:1 weight ratio. A certain amount of *n*-hexane was added to dissolve the precursor, stirred for 3–5 min, and deaerated via vacuum for 5–10 min to remove the trapped air. The mixture was then poured over the leaf template and cured at 70 °C for 4 h. Finally, the cross-linked silicone elastomer block was swollen by immersion in chloroform solution for 1 h to thoroughly detach the leaves from the cured Sylgard-184. The sample with a thickness of approximately 1 mm was dried at room temperature. Microstructures complementary to the surface morphologies of *Trifolium* and *P. tricuspidata* leaves were obtained (Figures S1 and S2).

2.3. Characterization of Biomimetic Micro-PDMS Pattered Surfaces. The surface morphologies of biomimetic micro-PDMS patterned surfaces were characterized by scanning electron microscopy (SEM, JSM5600LV, Japan). The water contact angles were measured using a DSA 100 optical contact angle measuring device (Kruss, Germany), and the measurements were carried out at room temperature. Contact angles were expressed as the average of three measurements at different positions on each substrate. A Nano Map 500 LS (AEP Technology company, USA) was used to measure the surface roughness (R_a , μm). The contact type of a very sharp probe vertically contacts the surface to be measured for lateral movement. The probe moves vertically along with the contour shape of the surface, and the tiny displacement is converted into electrical signals, which are amplified and processed to obtain the R_a . At least three different spots were determined on each substrate. The scan distance was set as 1000 μm , and 20 mg of contract force was applied.

2.4. Cell Culture In Vitro. Human cervical cancer (HeLa and SiHa) cell lines (ATCC) were chosen for the studies. Cells were cultured in an RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Sera Pro, USA), 1% HEPES (Sigma-Aldrich), 100 units/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin in culture flasks. Both cell lines were cultured under standard culture conditions: incubation at 37 °C in a 5% CO_2 gaseous environment and 95% humidity conditions.

2.5. Scanning Electron Microscopy (SEM). SEM was used to observe the morphologies of the HeLa and SiHa cells cultured on or adhered to flat PDMS and the resultant two micropatterned PDMS surfaces. The samples were cut into 1 cm diameters, sterilized with 75% ethanol for 30 min, and washed with PBS buffer solution three times for 5 min each time. HeLa and SiHa cells were seeded on the three different surfaces at a concentration of 20,000 cells/well in 24-well plates. After incubation for 4 and 24 h, the two kinds of cervical cancer cells were fixed to the samples with 2.5% glutaraldehyde at 4 °C for 4 h, dehydrated in an ascending ethanol series (30, 50, 75, 95, and 100% three times), air-dried, and sputter-coated with gold for observation by SEM.

2.6. Immunostaining. To analyze local adhesion, the expression of actin filaments was visualized by immunofluor-

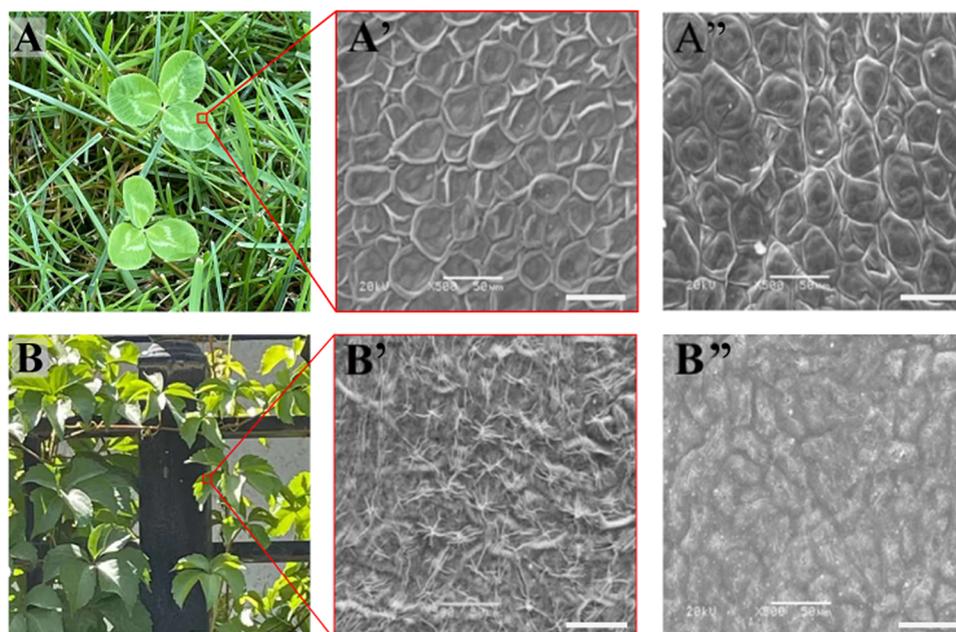


Figure 1. Original Trifolium leaf (A) and its SEM image (A'), original *P. tricuspidata* leaf (B) and its SEM image (B'), and Trifolium- and *P. tricuspidata*-templated PDMS surfaces (A'', B''); scale bars, 50 μm.

escent staining. As mentioned, HeLa and SiHa cells were seeded and incubated for 24 h. Then, 3.7% formaldehyde solution was added to fix the cells at room temperature for 30 min. After removing the fixative, the cells were incubated with a 0.5% solution of Triton X-100 at room temperature for 5 min followed by rinsing with PBS buffer. To visualize the actin filaments, the samples were incubated with a CytoPainter Phalloidin-iFluor 488 Reagent (50 μg/mL in PBS, Molecular Probes) for 60 min and washed again with PBS. The cell nuclei were stained with DAPI (50 μg/mL in PBS, Sigma-Aldrich) for 10 min followed by washing in PBS buffer three times. Laser confocal microscopy was applied to observe and take photos.

2.7. Statistical Analysis. The sizes of the cytoskeleton and nucleus after staining were measured according to micrographs of at least 20 spreading cells using ImageJ software. All data were analyzed by SPSS and expressed as the mean ± standard deviation (SD). The statistical significance between groups was set as $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Characterization of Micropatterned PDMS Surfaces. The morphological characterizations of the micropatterned PDMS surfaces were observed by a JSM-5601LV SEM machine (Figure 1). The samples were produced by mimicking the surface microstructure of leaves of Trifolium and *P. tricuspidata* with the template duplication method.

SEM images (Figure 1A') indicate that the surface microstructures of the original Trifolium (Figure 1A) and *Parthenocissus tricuspidata* leaves (Figure 1B,B') appear to be island-like and strip-like, respectively. The island-like patterns templated by Trifolium possess an average length of 40 μm and width of 30 μm for each periodic array of cells and exhibit a high degree of symmetry in Figure 1A''. As shown in Figure 1B'', there are several periodic arrays of radial-type strips with a length of 30 μm and width of 20 μm on each cell of microstructured PDMS-based biomimetic *P. tricuspidata*. The

surface microstructures of biomimetic microstructured PDMS are remarkably complementary to the original leaves.

It is well known that wettability is one of the most significant factors to influence the cell behavior; thus, the surface contact angles of the original leaves and micro-PDMS patterned surfaces were assessed by a DSA 100 optical contact angle measuring device (Kruss, Germany). As shown in Figure 2A,B,

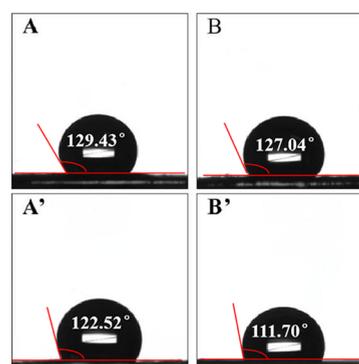


Figure 2. Photos of water droplets after contact on the (A) original Trifolium leaf, (B) original *P. tricuspidata* leaf, and (A', B') Trifolium- and *P. tricuspidata*-templated PDMS surfaces.

the water contact angles for Trifolium leaves and *P. tricuspidata* leaves were 129.43 ± 0.01 and $127.04 \pm 0.32^\circ$, respectively, which were surprisingly similar and hydrophobic, indicating that the flat PDMS (with a contact angle of $\sim 110^\circ$; Figure S3) with surface micropatterning modification was a more hydrophobic surface. In Figure 2A',B', the water contact angles were 122.52 ± 0.11 and $111.7 \pm 0.51^\circ$, corresponding to micro-PDMS templated by Trifolium and *P. tricuspidata* leaves, respectively. The original Trifolium and *P. tricuspidata* leaves are hydrophobic, and the water contact angles of the two micropatterned PDMS substrates are higher than that of the completely flat substrate but lower than those of their own native templates of leaves.

3.2. Cell Behavior Analysis. Micropatterned surfaces with distinct feature sizes and geometric structures have become new platforms for investigating the interaction between cells and substrates.^{29–31} Thus, micropatterned surfaces can be further utilized for evaluating how biophysical properties affect cell behavior and providing theoretical guidance for researchers to understand the specific behavior and state of cells in vivo. Hydrophobic PDMS and other hydrophobic surfaces can affect cell adhesion behavior and morphology.^{23–25} This suggests that cells tend to prefer hydrophilicity over hydrophobic surfaces by exhibiting more rounded morphologies, a lower degree of spread, and lower cell densities with increased surface hydrophobicity. Moreover, the Ti substrate with increasing wettability was proven to promote MSC proliferation, osteogenesis, and angiogenesis.^{32,33} However, it has been established that cells can interact with hydrophobic PDMS substrates even without any modification with ECM proteins.^{34,35} Herein, we investigated the response of cancerous cells to micropatterned PDMS substrates without any protein modification.

SEM analysis of the adhered HeLa and SiHa cells after culturing onto the two different micropatterned surfaces for 4 and 24 h provided qualitative information regarding the influence of the micropatterns on the cell morphology (Figure 3). The morphology of a single cell on a flat PDMS surface as a

negative control is shown in Figure 3A(a,d),B(a,d). After 4 h of culture, the morphology of HeLa and SiHa cells that adhered to the patterned PDMS surfaces templated with *Trifolium* is shown in Figure 3b,c,e,f. HeLa cells attached to island-like patterns and tended to stretch out their protrusions or pseudopodiums branching toward the proximal two-island-like patterns of *Trifolium* (Figure 3A(b)). Ghibaudo et al. observed similar results, in which NIH-3T3 fibroblasts on PDMS substrates with microscale pillar-patterned structures showed a branched morphology.³⁶ In addition, SiHa cells spread and covered the island-like substrate (Figure 3A(e)). The HeLa and SiHa cells on the flat PDMS surfaces were attached but did not spread well, as shown in Figure 3A(a,d). The cells adhered to the stripe-like patterns on PDMS surfaces templated with *P. tricuspidata* that exhibited different cell morphologies compared with those on the *Trifolium* patterns and the flat PDMS substrates. As shown in Figure 3A(c,f), HeLa and SiHa cells tended to directionally spread out. It is likely that cells spread along the direction of the microstripe pattern on the PDMS surface. HeLa cells spread elongated and exhibited few microextensions (Figure 3A(c)), while SiHa cells spread well and appeared to have more microextensions, and their protrusions were spread out from the main cell body along with the direction of strips and were easily observed (Figure 3A(f)), indicating that the cervical cancer cells had succeeded in attaching and spreading on the micropatterned surfaces.

After being cultured for 24 h, the HeLa and SiHa cells adhered to *P. tricuspidata* patterned PDMS surfaces and underwent different reactions by covering two adjacent strip-structure units or between two nearby units. HeLa cells had spindly shapes and apparent connections of cellular protrusions (Figure 3B(c)). However, SiHa cells were observed to have a flattened morphology with numerous microextensions, as shown in Figure 3B(f). Representative images of HeLa and SiHa cell adhesion behavior on *Trifolium* island-like surfaces are depicted in Figure 3B(b,e). In particular, a single HeLa cell attached and extended into two umbrella-shaped protrusions that roughly covered each half of the adjacent two-island microstructures (Figure 3B(b)). Some SiHa cells could cover only one island and take almost the entire island size, having a larger cell size (Figure 3B(e)), while the cancerous cells on flat PDMS surfaces attached and spread randomly, as shown in Figure 3B(a,d). HeLa and SiHa cells strongly modified their morphology to adjust themselves to microstructured PDMS by means of protrusions from the cell body. This morphology markedly implies that HeLa and SiHa cells can adhere and spread well on the micropatterned PDMS surfaces.

The difference between the patterns mimicking *Trifolium* and *P. tricuspidata* leaves can also be explained by considering the Ra of the two substrates. In comparison with the flat PDMS substrate, the two microstructured PDMS substrates provided more binding sites for the adhesion and extension of HeLa and SiHa cells. The RMS (root mean square) roughness values of the two biomimetic microstructures of PDMS were evaluated and are given in Figure 4. This reveals that the Ra of the patterned PDMS substrate fabricated with *Trifolium* as the template is approximately 1 1/2 times larger than that of *P. tricuspidata*-templated microstructured PDMS. Thus, the rougher surface of the *Trifolium*-templated substrate provides more contact sites for cell spreading than *P. tricuspidata*-templated and non-patterned PDMS substrates. The morphological difference between HeLa and SiHa cells implies that the

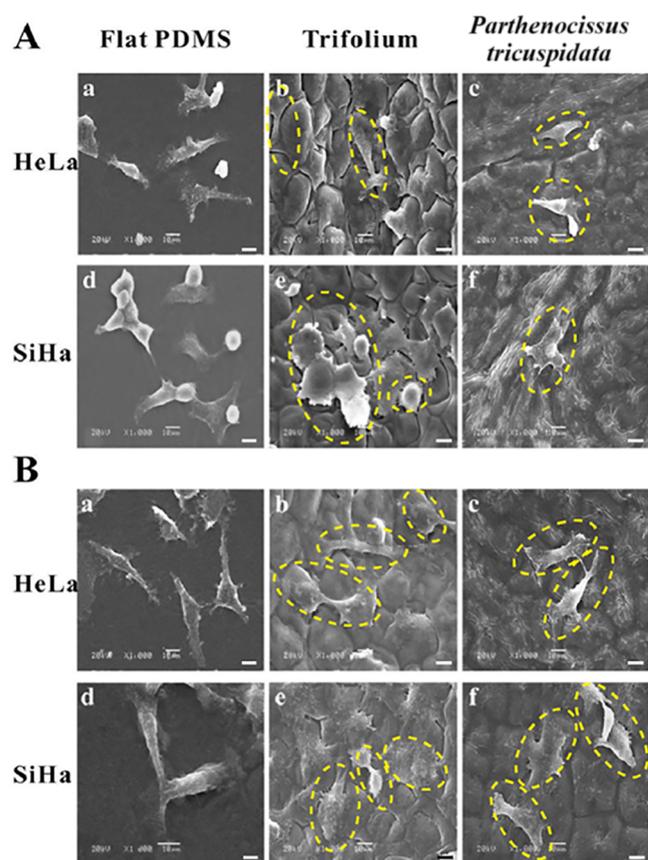


Figure 3. Influence of microstructured PDMS on the cell morphology of cancerous cervical cells. SEM images of the cell morphology of HeLa (a–c) and SiHa (d–f) cells seeded on (a, d) flat PDMS and micropatterned PDMS templated with (b, e) *Trifolium* and (c, f) *P. tricuspidata*, respectively. Images were taken after 4 h (A) and 24 h (B) of incubation. Scale bars, 10 μm .

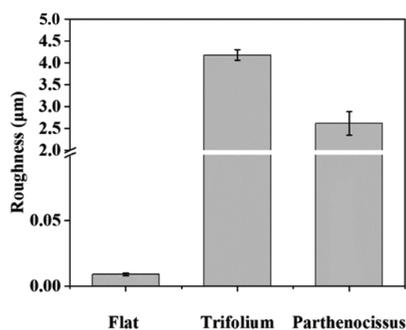


Figure 4. Surface roughness (Ra) of biomimetic micropatterned PDMS surfaces.

Ra and the geometry of biomimetic patterned substrates play a crucial role in inducing distinct morphological changes and cytoskeletal organization in the cells.

To further investigate the morphologies of HeLa and SiHa cells in detail, after 24 h of culture on distinct microstructured PDMS substrates, actin and nuclei were stained with CytoPainter Phalloidin-iFluor 488 and DAPI, respectively. Such cytoskeletal staining was performed at room temperature in the dark, and the corresponding confocal images are shown in Figure 5. Actin filaments were less developed on the flat PDMS substrate (Figure 5a). On the micropatterned PDMS substrates, the actin filaments of HeLa and SiHa cells displayed well-developed stretched fibers, which demonstrates a great spread of HeLa and SiHa cells on the patterned surface (Figure 5b,c). HeLa cells seem to recognize the size of the micropatterned PDMS surfaces templated with Trifolium.

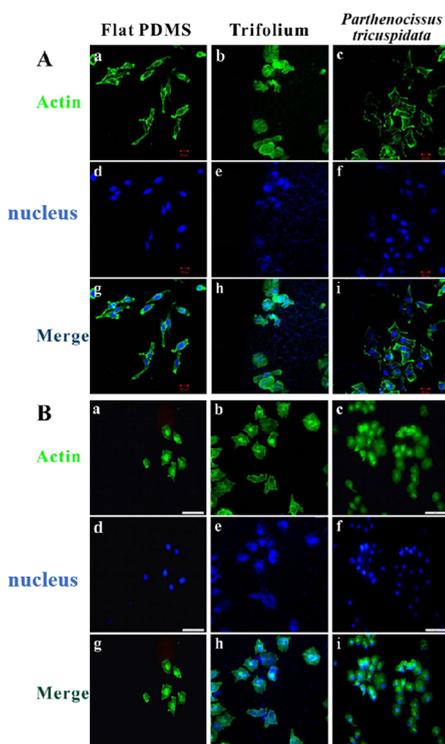


Figure 5. The cytoskeleton of HeLa and SiHa cells was observed by laser confocal scanning microscopy. HeLa (A) and SiHa (B) cells seeded on (a, d, g) flat PDMS and micropatterned PDMS templated with (b, e, h) Trifolium and (c, f, i) *P. tricuspidata*, respectively. Images were taken after 24 h of incubation at a magnification of 20X.

SiHa cells on the patterned surfaces of Trifolium appear round and widely spread. In contrast, on the non-patterned PDMS surfaces, HeLa and SiHa cells were observed to form a normal architecture morphologically, similar to the culture in plastic flasks. Thus, micropatterned surfaces can affect cell-substrate interactions, which ultimately cause changes in the actin cytoskeleton structure and distribution. As shown in Figure 5A, HeLa cells on the island-like patterns from Trifolium leaves were round in shape and exhibited a larger extension area than that on the flat substrate. The cytoplasm of HeLa cells was stretched, which resulted in a stripped shape and symmetric distribution. The cellular nuclei were round and located in the center of the cytoplasm. This illustrates that Trifolium island-like structures can significantly promote the development of the cytoskeleton but do not strongly affect the cell nucleus. Notably, the nuclear morphology seemed to be affected by the Trifolium-templated surfaces, as observed in the DAPI-stained panel. This is attributed to overexposure or nonthorough washes. This morphology elucidates that HeLa cells can adhere to island patterns using cytoplasmic spread. However, on the *P. tricuspidata*-templated PDMS substrates (Figure 5A(c,f,i)), HeLa cells were flat and exhibited a larger cell spreading area than that on the flat PDMS substrates. As shown in Figure 5B, similar to HeLa cells, the actin cytoskeleton of SiHa cells was round and had a larger stretched shape on the two biomimetic micro-PDMS samples than on the control substrate. The results indicate that SiHa cells can adhere to the surface using cytoplasm spread, and their morphologies indicate that the biomimetic micro-PDMS surface is an adaptive surface for HeLa and SiHa cells.

Micropatterned substrates have a great influence on cell behaviors, including cell morphological changes and cell function and activity changes,^{37,38} and actin is of great importance in transmitting extracellular forces to cells via integrins and plays an essential role in inducing signal transduction for cell function.^{39,40} Cells encounter various extracellular forces with the substrates they adhere to, which will eventually affect the cell stress state and activities. Briefly, the results show that biomimetic microstructured PDMS can be a well-defined surface for cell adhesion and spreading by the morphology of the cytoplasm and nucleus even just after 1 day of culture.

According to the cell image analysis, the cell nuclear/cytoplasmic ratios (N/C ratios) of both HeLa and SiHa cells were calculated. As shown in Figure 6, the N/C ratios of SiHa and HeLa cells on the two patterned substrates are significantly lower than those cultured on the flat PDMS surface, which illustrates a significant increase in cell size and cell spreading area, revealing that micropatterned PDMS can facilitate the attachment and extension of SiHa and HeLa cells.

4. CONCLUSIONS

In conclusion, micropatterned PDMS surfaces mimicking Trifolium and *P. tricuspidata* leaf surface morphologies were successfully developed by using the duplication method taking natural leaves as templates. In vitro cell culture studies with the resultant micropatterned PDMS surfaces using HeLa and SiHa cells demonstrated that the micropatterned substrates had a great influence on cellular behaviors, including an increase in cell adhesion, changes in morphology, and promotion of cellular extension. It was also found that there were significant differences between HeLa and SiHa cells in morphology on the two distinct patterned substrates, indicating that the cell

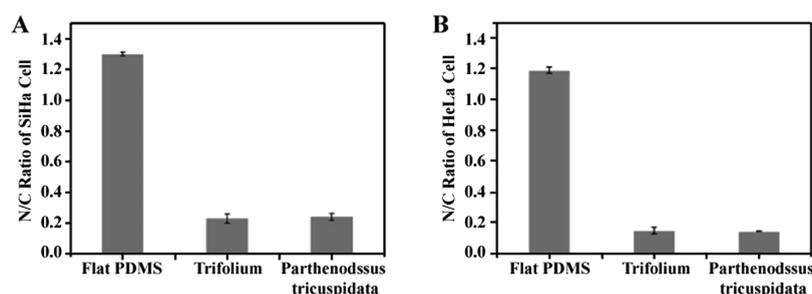


Figure 6. Cell nuclear/cytoplasmic ratios of (A) SiHa and (B) HeLa cells on flat and two micropatterned PDMS substrates.

response depended on the geometry and dimension of the micropatterned PDMS surface as well as the Ra. Compared with flat PDMS and plastic surfaces, biomimetic patterned surfaces are capable of inheriting the physicochemical properties of natural biological surfaces, which may be beneficial for cell culture. It is believed that micropatterned PDMS surfaces duplicated from natural leaves without any surface treatment by chemical and biological reagents will become new platforms for investigating the interaction between cells and substrates.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.2c01703>.

Figure S1, plant leaf that immersed into the PDMS precursor and initial morphology of the PDMS surface; Figure S2, digital images of the micropatterned PDMS surface with a diameter of ~ 15.0 mm, side view, and a thickness of ~ 1.0 mm; and Figure S3, water contact angle of the flat PDMS substrate (PDF)

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Author Contributions

Conceptualization: B.L., Y.Z., and X.W. conceived and designed the experiments; X.Z. and T.Z. prepared the material and performed the characterization of the data; X.Z. and Z.J. wrote the paper. All authors have read and agreed to the published version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was funded by the Gansu Provincial Science & Technology Department (20JR5RA558 and 20JR10RA055), National Natural Science Foundation of China (51775538 and 52005484), Lanzhou Science and Technology Development for Health (2019-034), and the Western Project of CAS.

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