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Role of feature curvature in contact guidance

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Abstract

This study examines the role of feature curvature in cellular topography sensing. To separate the effects of feature size and curvature, we have developed a method to fabricate grooved substrates whose radius of curvature (r) is varied from under 10 nm to 400 nm, while all other dimensions are kept constant. With increasing r up to 200 nm, mouse embryonic fibroblasts increased their spread area, but reduced their polarization (aspect ratio). Interestingly, on features with an r of 200 and 400 nm - where there was very little effect on spreading area and polarization - we find that internal structures such as stress fibers are nevertheless still strongly aligned to the topography. These findings are of importance to studies of both tissue engineering and curvature sensing proteins.

Keywords

Cell – substrate interaction; Surface topography; Cell spreading; Cell morphology; Fibroblasts

1. Introduction

The physical properties of a cell's environment play a large role in determining cell behavior and phenotype: the interplay between physical and biochemical signals influences responses that regulate cell growth, differentiation, shape change and cell death [1]. Within the field of mechanosensing, one area of particular interest is cellular interactions with surface topography: numerous studies have shown that cells react to underlying topographical features like grooves and ridges by modifying their cytoskeleton and aligning to the topography. This phenomenon is referred to as contact guidance [2].

A great deal of recent work has explored contact guidance in a variety of systems. Neuronal cells, epithelial cells, keratocytes and smooth muscle cells [3-6] all exhibit contact guidance on grooves and ridges by polarizing along the features. Fibroblasts respond both to feature size and feature density [7]. Furthermore, it has been shown that human corneal epithelial cells can elongate and align to ridges with widths as small as 70 nm [4]. Loesberg et al [8] showed that fibroblasts respond to grooved patterns with a height and width of 35 nm and 100 nm, respectively. Fibroblast and neurons plated on Ni nanowires for 24 and 72 h

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respectively display contact guidance [9]. Moreover, substrate topography has also been shown to influence cell differentiation. Human mesenchymal stem cells (hMSC) on polymethyl methacrylate (PMMA) nanopit arrays produced bone specific extra cellular matrix (ECM) proteins, despite the absence of osteogenetic supplements [10].

One of the mechanical factors that has received little attention to date is the curvature of features in the external environment: only a few studies have examined the effect of curvature on cell mechanotransduction. In one study, it was reported that the amount of tissue deposited is proportional to the local curvature [11], a finding that could be important in the field of tissue engineering and designing artificial implants. Endothelial cells on curved surfaces respond to flow rapidly, with marked changes in filamentous actin central stress fiber formation [12]. When rat melanoma cells are exposed to microcontact printed geometries with local curvature, there is strong localization of actin based cytoskeletal structures on the adhesive islands [13]. Herrera et al [14] used a multiscale modeling approach and reported that increased curvature leads to a higher inhibition of contractile force. In the previous experimental work, the structures used had radii of curvature on the micron scale and above. However, because cells in the body spread on ECM fibrils with diameters between 260 and 410 nm [15], it is important to examine the role of feature curvature in this size range.

In this work, we explicitly examine the role of curvature in contact guidance. In order to isolate the effects of curvature from other factors, we have developed a technique to create features with nominally identical dimensions but varying radius of curvature. The technique used to fabricate these features is shown in Fig. 1(a). Briefly, photolithography and plasma etching were used to generate an array of sharp lines with width w' , height h , and pitch p on a fused silica substrate. Next, a layer of silicon dioxide of thickness r was conformally deposited onto the substrate to give rounded features with width $w = w' + r$, radius of curvature r , and height h . By using starting patterns with appropriate w' , a series of substrates with identical w , h , and p could be generated, with only r varying from substrate to substrate. The details of the process are given below in the fabrication section.

We note that choosing the proper material for the substrate is crucial since the low optical contrast of cells requires a transparent substrate for transmission microscopy. Because glass does not etch uniformly, fused silica (170 μm thick) wafers were used [16]. In addition it also ensured that any effect we observe is only due to change in geometry and not due to rigidity changes, which could be the case for other commonly-used transparent materials such as silicone elastomers.

Using the above substrates, we analyzed the effect of curvature on cell morphology. Cell area and aspect ratio were examined on various substrates, and immunostaining of focal adhesions, stress fibers and microtubules were used to show the effect of curvature on these cytoskeleton components. We observe that feature curvature has a profound effect on both cell morphology and cytoskeleton organization. Specifically, increased radius of curvature decreases cell polarization. Using this technique, it may be possible to engineer precise geometries that can lead to better design of scaffolds and biomaterials for tissue engineering

2. Materials and methods

2.1. Fabrication of substrates

For this study, two sets of samples with fixed width and pitch were made. The first set consisted of sharp substrates ($r < 10$ nm) with varying height; the second set consisted of substrates with two fixed heights, and varying r .

Fabrication of sharp substrates: RCA cleaned fused silica wafers were covered with a 170 nm layer of organic bottom anti reflective coating (BARC) (Brewer Science) baked at 180 °C for 60 s on a hot plate. SPR 700 1.2 L (Shipley) resist was then spun on the substrates, and soft baked at 90 °C for 60 s. A layer of BARC before the resist helps in light absorption and destructive interference at the resist/BARC interface, this leads to a vertical side – wall profile.

The wafers were patterned by UV photolithography in a 5:1 i – line (365 nm) reduction stepper (GCA Autostep 200), postexposure-baked at 115 °C for 60 s, and manually developed for 60 s with AZ 300 MIF (AZ Electronic Materials USA) developer. To remove any leftover resist and ARC, a 75 s O₂ plasma descum was performed. With the patterned resist as a mask, the fused silica was etched by reactive ion etching (RIE) (Oxford PlasmaLab 80+), using O₂ >(2 sccm) and CHF₃ (50 sccm) gases at 40 mTorr and RF power of 240 W. Etching times from 1.7 minutes to 34 minutes were used to fabricate substrates with heights from 50 nm – 1 µm.

Fabrication of curved substrates: Because of the need for smaller initial linewidths, high-resolution OIR 620-7i (Arch Chemicals) resist was used. The resist was patterned and developed as above. Samples with line widths from 500 nm – 1.2 µm were generated. The samples were etched to two heights, 200 and 400 nm, followed by resist strip and cleaning. Finally, SiO₂ was deposited on the features by plasma assisted chemical vapor deposition (GSI PECVD) with gases N₂, SiH₄ and N₂O at 400 °C. This process results in conformal deposition and rounding of the features to a controlled radius of curvature approximately equal to the thickness of the deposited oxide.

2.2. Surface Characterization

Scanning electron microscopy (SEM) (Hitachi 4700) and atomic force microscopy (AFM) (XE – 100, Park Systems) were used to characterize the substrates (Supplementary Information Figure S1). Figures 1b-e show cross-sectional SEM images of substrates with $h = 400$ nm. As designed, the samples have constant $w = 1.3$ µm and r varying from <10 nm to 400 nm.

2.3. Cell Culture

The samples were silanized with 99.9% Hexamethyldisilazane (Sigma – Aldrich) and then coated with human plasma fibronectin (10 µg ml⁻¹; Roche) for 1.5 h at 37 °C and 5% CO₂. Mouse Embryonic Fibroblasts (MEF) were maintained in DMEM medium (Gibco) supplemented with 10% FBS (Gibco), 1% L-glutamine and 100 IU/mg penicillin-streptomycin (Invitrogen) at 37 °C and 5% CO₂. MEFs were plated 24 h before an

experiment at 80,000 cells per 1.5 cm² tissue culture dish, harvested and added to substrates at 100 000 cells/ml in ringer solution (150 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 20 mM Hepes and 2 g/l glucose, pH 7.4). Each of the petri dishes was filled with 2.7 ml of ringer solution and 0.3 ml of cells in ringer, i.e. 30,000 cells were seeded on the substrate. Cells from passage 12 – 17th were used during the experiments.

2.4. Immunofluorescence Staining

Fibroblasts were seeded onto the substrates. On the first set of substrates (sharp features with varying heights), the cells were allowed to spread for 75 min. On the second set of substrates (variable r) the spreading time was 150 min. After the spreading period, the cells were fixed with a solution of 4% paraformaldehyde in PBS preheated to 37 °C for 10 min. After fixation the samples were treated with a blocking/permeabilizing solution of 1% BSA/PBS for 1 h at room temperature followed by the addition of anti paxillin (B.D. Biosciences) and anti tubulin (gift from Prof. Chloe Bulinski) primary antibody (1:500 in 1% BSA/PBS) for 2 h. Next the samples were rinsed for 5 min three times with PBS followed by a 5 min rinse with antibody dilution buffer. After this the substrates were stained overnight with secondary antibody for paxillin (AlexaFluor 488 Invitrogen; 1:500 in 1% BSA/PBS) and microtubules (AlexaFluor 555 Invitrogen; 1:1000 in 1% BSA/PBS), additionally cells were also stained for F - actin with AlexaFluor 633 phalloidin (Molecular Probes). Phalloidin labeling was performed together with the secondary antibody labeling. Each of the samples was then rinsed for 5 minutes with PBS four times. Finally the substrates were coated with 100 µl of ProLong Gold Antifade Reagent (Invitrogen) to suppress photobleaching. Stained cells were imaged using a 40 ×, 1.35 NA oil objective (Olympus). An average of 20 – 40 and 50 – 70 cells were analyzed on each of the sharp and curved substrates respectively and 3 – 5 independent experiments were performed for each condition.

2.5. Time-lapse microscopy of live cells

Differential interference contrast (DIC) microscopy was used for live observation of cells on the patterned substrates. The substrates were glued onto the bottom of the 35 mm falcon petri dish with a hole in the center. Time-lapse micrographs were recorded with a 20 ×, 0.7 NA air objective (Olympus) through a cooled CCD camera CoolSNAP HQ (Roper Scientific Inc.) using Simple PCI software (Compix Inc.). Images were captured every 5 seconds.

2.6. Cell-to-substrate interaction using Scanning Electron Microscopy

SEM analysis was performed to determine whether the cells were lying on top of the features or conforming to the patterns. For this analysis, the cells were fixed using 0.1% glutaraldehyde (Calbiochem) for 60 s. After fixation the cells were dehydrated in a graded series of chilled ethanol (50%, 60%, 70%, 90% and 100%) and then critical point dried with liquid CO₂. Dried substrates were sputter coated with 10 nm Au-Pd and observed using an electron microscope (Hitachi 4700) at an accelerating voltage of 5 kV. In both cases i.e. sharp and curved substrates the cells were on the ridges and did not dip into the grooves.

3. Results

3.1. Fabrication of substrates with controlled radius of curvature (r)

We have developed a fabrication technique to precisely control the radius of curvature of the edges of ridges. Using this technique two sets of samples were prepared with the following dimensions.

(i) $h = 200$ nm; $r = 0$ ('sharp'), 50, 100, 200 nm, $r = \infty$ (flat).

(ii) $h = 400$ nm; $r = 0, 50, 100, 200, 400$ nm, $r = \infty$.

In all samples the feature width was 1.3 μm , close to the size of a single focal adhesion and the pitch was 3 μm . We note that the nominal value of $r = 0$ ('sharp') substrates is for samples with only the as-patterned features, which have a true radius of curvature below 10 nm. The geometry of the substrates was verified using SEM (width and pitch) and AFM (height). This method of controlling r is very precise and reproducible with no more than $\pm 10\%$ error. Additionally, the area patterned with oxide is surrounded by planar smooth area covered with the same material and has similar average roughness (Supplementary Information Figure S2), allowing for analysis of cellular behavior in a control condition and on topographic features simultaneously. This is the first demonstration of the fabrication of structures in which radius of the edges of ridges is independently controlled on the nm scale for the study of its effect on cellular mechanotransduction. For a given height other dimensions like width and pitch have been kept constant and only the edge radius has been varied.

3.2. Analysis of cell morphology on sharp substrates

The analysis of cell morphology was done in 2 steps. First, cells were plated for 75 min on sharp substrates with varying heights (50, 100, 200, 400, 600, 800 and 1000 nm). This time period is enough to induce morphological changes in the cell and allowed sufficient time for the cells to stabilize from the active spreading phase [confirmed by time series analysis shown in supplementary information Figure S3]. After this the cells were fixed and stained for F - actin as described in section 2.4. The shape of the cells was determined by drawing a best-fit ellipse around the cells using a standard Image J plugin [National Institutes of Health (NIH)]. The degree of cellular polarization was quantified by calculating the anisotropy ratio (AR), defined as the ratio of length of the cell parallel to the features to the length perpendicular to the ridges. As shown in Fig. 2, the AR is close to 1 up to $h = 100$ nm, then increases to a value of ~ 3 -4 for $h = 400$ nm, above which it is roughly constant. Therefore we identify $h = 100 - 200$ nm as the threshold for contact guidance in this system. The observed response to sharp substrates was in accordance with previous studies [16-18] where it has been reported that AR increases with the height of grooves and ridges. For prolonged spreading it has been reported that the threshold height for alignment is in 35 – 75 nm range [8]. We note that the AR also increases with decreasing feature width [19], although this parameter was not examined here. Movies (S1 and S2) of cell spreading on 50 nm and 600 nm high features are shown in the supplementary information.

3.3. Analysis of cell morphology on curved substrates

Based on the above results, samples with $h = 200$ and 400 nm, and varying r , as described above, were used to examine the effects of curvature. Using these substrates, we investigated the effect of r on cell area and AR (Fig. 3). The spreading time on these substrates was 150 min, which allowed sufficient time for the formation of focal adhesions. On both heights, the spread area increases with r , and is higher on flat substrates than on substrates with patterned lines (Fig. 3a, b). On 200 nm high features, the spreading area showed an increase from $2500 \mu\text{m}^2$ to $3630 \mu\text{m}^2$ as r increased from ~ 0 to 200 nm. On 400 nm high features the spreading area showed an increase from $2400 \mu\text{m}^2$ to $3400 \mu\text{m}^2$ as r increased from ~ 0 to 400 nm. One-way ANOVA tests revealed that both of these changes were statistically significant ($p < 0.001$). The maximum change in spread area for both heights is reached at $r = 200$ nm, after which the change is statistically insignificant ($p > 0.75$).

Movie S3 and S4 show cell spreading on 200 nm high features with $r \sim 0$ nm and $r = 200$ nm. A clear change in the morphology can be observed. For both 200 nm and 400 nm feature heights, the AR decreased smoothly with increasing r . On 200 nm high substrates, the AR decreased from 3.3 on sharp features to 1.7 on features with $r = 200$ nm. On 400 nm high features, the AR decreased from 4.8 on sharp surfaces to 2.4 on features with $r = 400$ nm. These changes were statistically significant ($p < 0.001$). The AR was 1.2 on flat substrates. Thus, increasing the radius of curvature, while maintaining the feature height, can cause the cells to assume a shape that is close to that observed on flat substrates. For 400 nm high features the AR decreases smoothly with radius of curvature up to $r = 200$ nm, after which the change is statistically insignificant ($p > 0.75$).

Interestingly, 400 nm high lines consistently ($p < 0.001$) showed larger AR than the 200 nm high features, at all values of same r . This observation raised the possibility that cells might contact the troughs of the grooves. To ensure that the cells are not dipping inside the grooves, scanning electron microscopy was used to precisely examine cellular morphology on the patterns. It was observed that cells attach themselves mainly to the top of the ridges and do not descend into the grooves, as shown in Fig. 4. This observation suggests that the radius of curvature of the tops of the ridges is what the cells are primarily sensing. Nevertheless, given the slightly greater polarization on the taller lines we cannot rule out minor contacts with the base of the features.

3.4. Cell cytoskeleton and focal adhesions

Fluorescent observation after 150 min of cell spreading revealed well-defined actin fibers and microtubules (Fig. 5). On the patterned substrates, the stress fibers and microtubules were polarized along the lines, whereas on a flat surface no such polarization was observed. On all substrates, microtubules originated from the microtubule organizing center (MTOC, near the nucleus), out to the cell periphery. The microtubules on both the sharp and curved substrates were partially aligned in the direction of pattern as shown in Fig. 6(b,e). On flat surface the cells took an isotropic shape with round stress fibers at cell periphery and diffused actin in the cytoplasm as shown in Fig. 6(g). On flat substrates microtubules seem to form a radiating network from the MTOC close to the nucleus to the outer edge of cell as shown in Fig. 6(h). On flat control surface numerous focal adhesions were found all around

the circumference of the cell as shown in Fig. 6(i). Whereas on sharp and curved surfaces the focal adhesions formed in the interior of the cell, on top of ridges (Fig 6 c,f). On sharp and curved surfaces the nuclei of the cells were aligned along the pattern whereas on flat surface a mix of randomly elongated and rounded nuclei were observed.

Interestingly, although cells on the $r = 200$ nm and 400 nm substrates had spreading areas and polarization that resembled flat substrates (Fig 3), these substrates nevertheless displayed robust focal adhesions and cytoskeleton alignment to the grooves (Fig 6 a,c,d,f).

4. Discussion

The present study is the first examination of the role of feature curvature in contact guidance where the radius of curvature is varied independently of other dimensions. We found that for $1.3\text{ }\mu\text{m}$ wide grooves separated $3\text{ }\mu\text{m}$ apart, features needed to be higher than 200 nm and their radius of curvature below 200 nm to evoke robust morphological responses. However, cytoskeletal and adhesive effects were seen at radii of curvature as high as 400 nm.

It has been shown that after 24 hours, fibroblasts can reorient to grooves as small as 35nm deep [8]. On our substrate, cellular morphologies to feature heights of 100 nm and lower approximated a flat surface, with an AR of approximately 1.5 (Fig 2). Two important differences from the previous study reporting greater sensitivity were the length of culture (24 hours versus 75 minutes in this study) and the width of the grooves (100 nm versus the $1.3\text{ }\mu\text{m}$ in this study). We investigated the effect at shorter times to better understand the effect of r on features 200 and 400 nm high due to the sharp transition in the polarization values (AR) at these two heights. We note that this range is similar size to the size of ECM fibrils [15] *in vivo*.

These results can help to clarify the contribution of various mechanisms in contact guidance. It has been proposed that the frequency of filopodia formation in the direction perpendicular to the features is lower because of the stress involved in bending the filopodia around a sharp corner, leading to cell polarization [20]. Although the cells in these studies more commonly show a smooth leading edge (lamellipodium) [Movie S1 and S2], curvature should modify the protrusion of lamellipodia in the same way as filopodia. These results suggest that bending stress begins to modify protrusion below a radius of curvature of ~ 200 nm, a prediction that can be directly studied in further experimental and theoretical work. A second proposed mechanism is confinement and alignment of focal adhesions to the top surfaces of patterns: focal adhesions orient themselves to the pattern, which in turn polarizes the actin filaments that originate from the focal adhesions, and thus the entire cell [21]. However, we observe that focal adhesions remain confined to ridges even for large r , where cell spreading is close to isotropic. Therefore, these results suggest that this mechanism alone cannot cause cell polarization.

Finally, the range of curvature sensing seen in this study will help to determine whether membrane-bound curvature-sensing proteins are involved in contact guidance. For example it has been reported that BAR (Bar/Amphiphysin/Rvs) domains are the sensors of membrane curvature [22-24]. Recently, Bhatia et al [25] examined eNBAR proteins on structures with

varying radii of curvature and found monotonic drop-off in binding up to $r \sim 100$ nm, after which the decrease slows. This cutoff in curvature is somewhat smaller than seen in our sample, indicating that different sensing mechanism may be at play here. However, the difference is not so large as to definitively rule out this mechanism.

Our findings are relevant for tissue regeneration. For a tissue to function/regenerate properly it is necessary that the building blocks i.e. individual cells be healthy. It has been reported that for individual cells to survive they need to have large spread area [26] and for a tissue to elongate polarized stress fibers are required [27]. The cells on the curved surfaces show both these conditions, and thus curved geometry could potentially be applied in designing better implants.

5. Conclusions

This is the first study where the radius of curvature was controlled precisely at the nanometer scale. In this study we demonstrated the fabrication process of a substrate with controlled radius of curvature from 50 nm to 400 nm. The features were fabricated in fused silica, which made sure that the cell response was due to the underlying geometry and not because of change in rigidity; which might be the case with elastomeric substrates. Furthermore early spreading (75 min) results reveal that the threshold height sensed by the fibroblasts for sharp substrates with groves and ridges, 1.3 μ m wide and 3 μ m in pitch was in the 100 - 200 nm range. Additionally, we also showed a definitive influence on cellular behavior and morphology due to controlled curvature and cells are able to sense and respond to radius of curvature over a wide range 50 – 200 nm. The potential application might be in the area of tissue engineering in particular where independent control of cell morphology and fiber alignment is required. Future work will be targeted at exploring the mechanisms that lead to curvature sensing by fibroblasts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Fabrication of substrates with controlled radius of curvature (r). (a) Schematic of the microfabrication process used to make the features with a given r . Drawings not to scale. (b,c,d,e) Cross-Section scanning electron micrographs of substrates with varying r , from 0, 100, 200 and 400nm. (Note that other dimensions like ridge width (w), pitch (p) and height (h) of the features is same in all the substrates). Scale Bar – 1 micron.

Figure 2. Effect of feature height on cell shape after 75 min of cell spreading. Error bars indicate mean $\pm$ Standard Deviation.

Figure 3.

Spread area and anisotropy ratio vs. radius of curvature. (a, b) Cell Spread area vs radius of curvature on 200 and 400 nm high substrates respectively. (c, d) Anisotropy ratio vs radius of curvature for 200 and 400 nm high substrates respectively. Cells were plated for 150 min. Asterisk indicates statistical significance relative to $r \sim 0$ nm ($p < 0.001$). Error bars indicate mean $\pm$ Standard Deviation.

Figure 4.

Cells spread on top of features. Scanning electron micrographs of cells on grooves and ridges with different radius of curvature. (a, b) MEF cells spreading on substrate with $h = 400$ nm and $r \sim 0$ nm. (b) An enlarged section of the image in panel (a) showing the cell lying on the ridges. (c, d) Cell spreading on substrate with $h = 400$ nm, $r = 400$ nm. Cells were fixed after 150 min and critical point dried. Cell marked with asterisks and substrate marked with arrows (white – ridges, black – grooves). Scale Bars - a and c, $30\ \mu\text{m}$; b and d, $5\ \mu\text{m}$.

Figure 5.

Cells stained for actin (blue), tubulin (red) and paxillin (green). (a) Cell on a substrate with $h = 200\text{ nm}$, $w = 1.3\text{ }\mu\text{m}$, $p = 3\text{ }\mu\text{m}$ and $r < 10\text{ nm}$. (b) Cell on substrate with same h , w and p as (a) but $r = 200\text{ nm}$. (c) Cell on flat substrate. Cells on sharp substrates align and elongate along grooves and ridges, cells on flat substrates are mostly round where as cells on substrate with a radius of curvature show a morphology in between that of sharp and control substrate. Cells were spread for 150 min. Arrow indicates the direction of pattern. Scale bar - $20\text{ }\mu\text{m}$.

Figure 6.

Fluorescent images of cytoskeleton of fibroblasts on sharp, curved and flat substrates after 150 min of spreading. On substrates with $r \sim 0$ nm (a) the stress fibers and actin are polarized. (b) Tubulin is aligned in the direction of grooves and ridges. (c) Focal adhesions were aligned along the ridges in the protruding lamellipodium. Fibroblasts on pattern with $r = 200$ nm exhibit a morphology between cells on substrate with $r \sim 0$ nm and flat surface, (d) Stress fibers were aligned in the direction of grooves and ridges and the actin network was well organized. (e) Microtubules were polarized in the direction of pattern. (f) The cells had significantly higher number of aligned focal adhesions as compared to cells on substrates with $r \sim 0$ nm. Cells on flat surface. (g) Actin cytoskeleton was well developed with circular bundles of stress fibers throughout the cytoplasm. (h) Microtubules in cells were well organized. (i) Focal adhesions were seen around the periphery of the cell. Arrow indicates the direction of grooves and ridges. Scale bar - $20 \mu\text{m}$.