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NANOPATTERNED INTERFACES FOR CONTROLLING CELL BEHAVIOR

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Abstract

Many studies have demonstrated that microscale changes to surface chemistry and topography affect cell adhesion, proliferation, differentiation, and gene expression. More recently, studies have begun to examine cell behavior interactions with structures on the nanoscale since *in vivo*, cells recognize and adhere to cell adhesion receptors that are spatially organized on this scale. These studies have been enabled through various fabrication methods, many of which were initially developed for the semiconductor industry. This review explores cell responses to a variety of controlled topographical and biochemical cues using an assortment of nanoscale fabrication methods in order to elucidate which pattern dimensions are beneficial for controlling cell adhesion and differentiation.

Keywords

Nanopatterns; nanotopography; cell adhesion; differentiation; surface modification

1. Introduction

In the fields of biomaterials and tissue engineering, the ability to modulate cell behavior on surfaces is essential, whether attempting to resist or promote cell adhesion on biomaterial implants, to direct cell growth and differentiation when creating *in vitro* engineered tissue, or to generate *in vitro* tissue models. The goal is often to generate surfaces that more closely mimic the extracellular matrix *in vivo*. Since cells are not exposed to a contiguous presentation of adhesion ligands in the body, specifically patterning either physical or chemical cues can create more biomimetic matrices. While chemical patterning of biomolecules at the microscale has been known for some time to modulate cell attachment, growth and differentiation, the importance of nanoscale interfaces has induced a recent surge towards exploring cellular responses to nanoscale patterns.^{1,2}

In vivo, cells recognize and adhere to cell adhesion receptors that are spatially organized on the nanoscale. The integrin, which is the most common cell adhesion receptor, is approximately 10 nm in diameter.³ Integrins also cluster to form focal adhesions, which are necessary for robust adhesion as well as intracellular signaling and typically occur in the

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range of 10–200 nm.^{4–7} This suggests that presentation of cell adhesion ligands at the nanoscale is necessary for mimicking the extracellular matrix and *in vivo* milieu, and in turn, directing cell adhesion and growth or differentiation.

Initial studies demonstrating the importance of presenting adhesion ligands on the nanoscale relied upon estimations and mathematic models for pattern size and spacing.^{8–12} More recently, fabrication methods, mainly those borrowed from the semiconductor industry, have allowed for precise control of nanoscale geometries, both in terms of topography and chemical patterns.¹³ This review explores cell responses to a variety of controlled topographical and biochemical cues using an assortment of nanoscale fabrication methods. Other techniques such as electrospinning also present nanoscale cues and have been recently reviewed.^{14,15} Herein, we cover surfaces, which have been precisely patterned at scales below one micron, in order to elucidate which sizes are beneficial for controlling cell behavior.

2. Common Patterning Methods

Recent progress in nanofabrication techniques has brought us closer to accurately imitating the scale of the natural *in vivo* extracellular environment. Electron beam (e-beam) lithography is one of the several micro/nano-fabrication techniques adapted from the semiconductor industry to create nanoscale patterns. E-beam lithography has been employed in a variety of approaches ranging from ablation of a material to crosslinking polymers. Traditionally, a substrate coated with an appropriate resist is exposed to an electron beam. The resulting substrate is then developed to create the desired nanotopography. Nanogrooves and other patterns with dimensions as small as 40 nm have been fabricated for cell studies using electron beam lithography.^{16–24} This technique offers design flexibility in not only physical parameters but also the ability to create highly customizable chemical patterns while sacrificing speed and overall pattern area.^{24,25}

Photolithography with polarized lasers or UV radiation is another method used to create the nanopatterns in the following studies by exposing a photoresist coated substrate to specific radiation through a photo mask. The substrate is developed, removing the unexposed portions and leaving behind a pattern. Traditional photolithography using UV radiation is typically limited by the wavelength but polarized lasers have been used to circumvent this limitation to create patterns with finer dimensions and features. Polarized lasers with low wavelengths have the potential of generating nanoscale structures that traditional photolithography methods are unable to create.^{26–32}

Other methods can utilize the patterns formed with electron beam lithography or laser irradiation as molds to transfer topographical or chemical patterns onto other substrates. Soft lithography, also referred to as nanoimprint and nanocontact lithography, employs stamps to transfer topographical or chemical patterns onto substrates. Nanoimprint lithography involves using a mold to press the desired pattern into a polymer heated above its glass transition temperature, while nanocontact lithography utilizes the same premise as microcontact printing, but with features below one micron.

Several techniques have exploited the self-assembly of materials to generate nanoscale patterns. Colloidal lithography is one such technique used to create repeating patterns on substrates with the aid of micro- or nanoparticles, which self-assemble into a monolayer on surfaces. Typically, this colloidal monolayer has been used as an etch mask to develop nanotopographies into the substrate material.^{33,34} Other self-assembly methods such as block copolymer micelle nanolithography have been used to pattern soft polymer surfaces. This technique relies on the self-assembly of block copolymers, which form a close-packed hexagonal template (Figure 1).

Using block copolymer micelle nanolithography, gold nanoparticles ranging from 2–15 nm in diameter can be patterned hexagonally onto glass,³⁵ and then functionalized with linker molecules (specific to the desired final polymer substrate). The surface of the glass is then coated with a polymer, and the linker molecules connect the gold nanoparticles to the final polymer substrate. The distance between particles can range from 15 to 200 nm depending on the molecular weight of the diblock copolymer. This novel fabrication method allows the flexibility to choose a variety of polymers (polystyrene, poly(dimethylsiloxane) (PDMS), and poly(ethylene glycol) (PEG) hydrogels of varying molecular weights) to pattern with nanoparticles. The study, design, and implementation of creating nanoscale patterns with current nanofabrication techniques is still improving and progressing. Below we discuss the progress of using the current techniques for influencing cell behavior.

3. Cell Response to Nanopatterned Surfaces

Most cell studies have utilized similar pattern features falling under the categories of grooves, columns, pits, or functionalized dot arrays. Each pattern is designed with a different purpose and will generally elicit unique cellular responses compared to unpatterned substrates. Depending on the pattern design and dimensions, the pattern may increase or decrease cell adhesion, influence alignment to patterns, or even alter genotypic expression.

3.1. Grooved/Ridged Substrates

Topographical patterns with grooves and ridges have been tested with varied results. One of the most apparent and immediate effects of nanogrooved surfaces on cell behavior is the alignment and elongation of cells along the axis of the pattern. Other effects of nanogrooved surfaces include but are not limited to alignment of actin fibers, changes in proliferation and cell density, and up or down regulation of the expression of specific proteins.

Various cell types cultured on grooved nanotopographical patterns generated using laser irradiation have shown cell body, actin fiber, and collagen matrix alignment orientated in the direction of the grooves as well as more elongation than the unpatterned control. The cells on the nanogrooves attained higher cell densities,^{26–28} and the effect of the patterns on cells also appears to be both cell specific and species specific. In a study using patterns created with laser irradiation, different cell types were cultured on grooves ranging 200–430 nm apart and 30–100 nm deep. Human embryonic kidney (HEK-293), Chinese hamster ovary (CHO-K1) cells, and skeletal myoblasts elongated along the direction of the structures but only when the periodicity was above a cell specific critical value. For the HEK-293 cells, no alignment was seen at 200 nm, and half of the cells were aligned at 270 nm. Cell alignment

was seen at periodicities of 340 and 430 nm after 24 hours, but after 48 hours, the cells were confluent and alignment was no longer observed. With CHO-K1 cells, alignment was seen at 6 hours at 340 and 430 nm, but weakly aligned at 270 nm. The rat skeletal myoblasts only demonstrated alignment at 430 nm, but human skeletal myoblasts aligned at 340 and 430 nm, and weakly at 270 nm for at least 4 days.²⁹ The pitch dimensions of the nanogrooves also appear to affect cell adhesion and proliferation. Cells cultured on a range of sub-micron nanogrooves were more stable in fluid shear flow than cells cultured on micron-scale patterns. The cells showed a significantly increased amount of adhesion and retention.^{36,37} Pattern dimensions can also influence the mechanical properties of cells cultured on 650 nm, 500 nm, and 332.5 nm wide nanogrooves. Vascular smooth muscle cells increased the measured ultimate tensile strength and Young's modulus within physiological normal values when cultured on nanoscale grooves.³⁸

Grooved patterns have also been generated through photolithography. Bettinger *et al.* used a GCA AS200 stepper to etch 600 nm wide and 600 nm deep grooves.³⁹ Although the endothelial progenitor cells (EPC) showed reduced proliferation on the nanostructured surface, these cells had increased mobility and congregated into large band-like structures aligned to the grooves. The EPCs cultured on flat surfaces were only able to form a confluent monolayer of cells. Capillary tubes induced by adding Matrigel formed on the nanogrooves and exhibited longer and more uniform organized structures in comparison to the short randomly orientated capillary tubes on the flat substrates.³⁹ Using a variation of UV photolithography, a UV-assisted capillary lithography technique was used to create nanogrooves in a PEG substrate. Neonatal ventricular myocytes were then cultured on patterns ranging from 50 nm to 800 nm wide and 200 to 500 nm deep. The myocytes were observed to be sensitive to the varying pattern dimensions, with 800 × 800 nm patterns resulting in greater cell area and Cx43 expression, and higher conduction velocity.⁴⁰

Soft lithography is another technique capable of quickly generating grooved patterns in various substrates. Eliason *et al.* combined the use of microimprint and nanoimprint lithography to produce polycarbonate nanoscale topographies on top of microscale patterns.⁴¹ Through multiple hot embossing steps, larger microscale grooves of either 2 or 10 μm wide were patterned with no grooves, parallel 50 nm grooves, or perpendicular 50 nm nanogrooves (Figure 2). MC3T3-E1 osteoblast-like cells seeded on the various substrates demonstrated a stronger dependence to align to the microscale patterns regardless of the parallel nanogrooves. However, the perpendicular nanogrooves decreased the number of cells aligned to the larger microgrooves by half. They also observed filopodia extending out from the cells along the perpendicular nanogrooves, which they speculated lead to the decrease in cell alignment along the microgrooves.

In another study, nanogroove patterns were created ranging from 100 to 400 nm width and 200 to 2000 nm pitch in poly(methyl methacrylate) (PMMA).⁴² The axons of mouse sensory ganglia preferred growing on the ridges rather than the gaps or grooves between the ridges. The distance between ridges did not appear to matter but wider ridges allowed more axons to spread on the ridge. Another group, Hu *et al.* produced patterns of ridges and grooves as small as 100 nm width and 200 nm height using nanoimprinting on polystyrene.⁴³ Patterns with 350 nm half-pitch ridges were shown to be more effective than 10 μm ridges in terms

of cell alignment and elongation. Bovine pulmonary artery smooth muscle cells seeded on the nanoscale pattern showed 92% alignment as opposed to 37% for the microscale pattern. Evidence suggested that deeper ridges of 800 nm lead to increased alignment of the cells. Martinez *et al.* studied the cell behavior of MG63 osteosarcoma cells on two distinct PMMA patterns: grooves that were 200 nm wide, 200 nm deep, and 200 nm apart; and nanopillars that were 300 nm wide, 30 nm tall, and 300 nm apart.⁴⁴ The cells cultured on the nanogroove pattern preferred to grow on the ridges rather than channels and formed focal contact points along the pattern. It was suggested that the cell membrane was unable to enter the nanostructures, and the focal adhesion sites organized linearly along the nanopatterned structure, causing elongation.

Russell *et al.* studied the effects grooves made with soft lithography, ranging from 400 nm to 4000 nm pitch with 300 nm deep grooves had on human trabecular meshwork (HTM) cell orientation.⁴⁵ A majority of the cells seeded on patterned substrates elongated and aligned to the grooves. Cells were cultured on grooves with 200 nm width, 200 nm apart, and 300 nm depth to study intracellular reactions to the patterns. F-actin filaments extended indiscriminately on smooth surfaces and in a parallel fashion on the patterned surface. Myocilin, a protein expressed in the trabecular meshwork, is lost when HTM cells are cultured on normal tissue culture plastic, yet an increased quantity of myocilin mRNA was observed in HTM cells on nanopatterned surface, which translates to an increase in myocilin protein concentration. Thus the concentration of myocilin in the cells on the patterned substrate was 12 times that of the cells on the smooth substrate. Yim *et al.* used nanoimprint lithography to transfer nanogrooves with dimensions of 350 nm deep, 350 nm width, and 700 nm pitch onto PDMS.^{46,47} A high percentage of the smooth muscle cells grown on the patterned substrate orientated with the nanopattern lines. Human mesenchymal stem cells were then studied under the influence of topographical and biochemical signals to induce cell differentiation. Although proliferation rates dropped, the cells cultured on the nanogrooves showed significantly more elongation and alignment compared to the unpatterned substrates. The presence of neuronal markers such as MAP2, β -tubulin III (Tuj1), and synaptophysin were also detected in the cells cultured on the nanopatterns with and without the addition of retinoic acid. Assorted changes in gene expression were also found in the cells on the nanopatterns; Yim *et al.* measured upregulation of neuronal markers SOX2, MAP2, neurofilament light peptide, and tyrosine hydroxylase, muscle markers myosin light chain and myf5, and vascular marker CD34.⁴⁶ Cells grown on unpatterned surfaces only expressed some of these markers with the presence of retinoic acid in the culture medium. Zhao *et al.* further increased the complexity of the study between cell interactions and nanogrooves by culturing human mesenchymal cells under low oxygen conditions. The cells cultured on the 350 nm width, 700 nm pitch, 250 nm depth nanogrooves under normal oxygen conditions exhibited alignment to the patterns as well as increased differentiation. The cells cultured at a 2% oxygen level were able to exhibit the same alignment to the nanogrooves but with reduced differentiation rates.⁴⁸ Finally, Lenhert *et al.* employed the Langmuir-Blodgett technique to first create regularly spaced grooves of different depths, either 50 nm or 150 nm, with an approximate width of 500 nm.⁴⁹ Then these nanotopographies were nanoimprinted onto a polystyrene surface. Calf primary

osteoblasts were cultured on the surfaces, and subsequently aligned parallel to the grooves. Cells that were cultured on the 150 nm grooved surfaces showed greater alignment.

Electron beam lithography is another technique commonly utilized to generate grooved patterns with materials ranging from gold to polyethylene glycol. Loesberg *et al.* used electron beam lithography to generate nanoscale grooves ranging from 20–1000 nm width, 40 nm to 2000 nm pitch, with equal spacing between grooves and ridges, in polystyrene.¹⁶ The depths of these patterns ranged from 4.4 nm to 353 nm. Rat dermal fibroblasts randomly spread across the smooth control as expected. Cells grown on the patterns with the deeper grooves, 80 to 350 nm, showed the most alignment to the pattern, and alignment decreased as the depth and pitch of the grooves decreased. Fibroblasts cultured on patterns with 35 nm depth randomly spread and showed no alignment difference from the smooth surface. Teixeira *et al.* used human corneal epithelial cells cultured on nanogrooves, and found these cells not only elongated but also extended lamellipodia along the direction of the nanogrooves (Figure 3).¹⁷ The depth of the grooves appeared to play a greater role than the pitch in guiding cell alignment, as cells seeded on 600 nm deep grooves showed greater orientation than on the 150 nm deep patterns. Cells cultured on smooth surfaces were more round and extended lamellipodia in random directions.

Goto *et al.* used electron beam lithography to generate grooved patterns as small as 300 nm width and 150 nm depth with spacing approximately 300 nm.^{21,22} A pattern was drawn by exposing a resist to the electron beam. The pattern was then developed and sputter coated with gold and titanium resulting in nanogrooved patterns, and 1-octadecanethiol (ODT) was attached to the gold surface to control wettability. Twice the number of mouse fibroblast 3T3 cells attached to the ODT functionalized patterns compared to the quartz controls.

E-beam lithography can also be used to create templates, which was done by van Delft *et al.*²³ Here, electron beam lithography was used with reactive ion etching to make nanogrooved master templates in silicon followed by replicating the patterns through solvent casting polystyrene. Through cell studies, van Delft *et al.* were able to demonstrate that rat dermal fibroblasts reach a point where there is no longer contact guidance. Random cell spreading occurs at a depth less than 70 nm or a width of 100 nm. Alternately, cells exhibited alignment to the nanogrooves at dimensions greater than 70 nm depths and 100 nm widths. It was speculated that this may be associated with the size of various contact molecules as the cells extend along the groove. Lamers *et al.* used both electron beam lithography and laser interference lithography to generate patterns with dimensions ranging from 40 nm to 1000 nm width and 10 nm to 153 nm depth. Using osteoblasts, results showed cell behavior similar to previous studies on other cell types. The alignment of osteoblasts was apparent down to a pattern width of 75 nm. Ordered calcium phosphate mineralization was also observed developing in line with the nanogroove direction.⁵⁰

There are several other less common techniques used to create nanogrooves. Nanoscale injection has been used to create nanogrooves of 450 nm wide, 190 nm deep, and 1000 nm ridges with polycarbonate and polycaprolactone.⁵¹ Fibroblasts and endothelial cells elongated down the groove and extended filopodia along the ridges. Martino *et al.* used masks in conjunction with plasma enhanced chemical vapor deposition method to generate

grid-like and grooved patterns. The hBM-MSCs cultured on the grooves showed elongation as well as alignment of F-actin fibers along the groove direction.⁵²

Progress has also been made in attempts to understand the reasoning behind cells aligning to nanotopographic grooves. Fujita *et al.* recently made an observation through time-lapse microscopy that filopodia extending perpendicular to the nanopatterns retracted far more quickly than protrusions extending parallel to the patterns.⁵³ Another recent study has suggested that while nanotopography and substrate stiffness play a role in determining a cell's mechanical properties, nanotopography plays a more dominant role in determining cytoskeletal organization and focal adhesion formation.⁵⁴

The use of nanotopography has also been combined with chemical patterning to produce interesting results. Charest *et al.* used a combination of nanoimprint lithography and microcontact printing to create a topographical pattern overlaid with chemical lanes.⁵⁵ They fabricated a nanoscale topographical structure consisting of nanogrooves using nanoimprint lithography. The pattern consisted of grooves with 100 nm depth, 100 nm width, and 200 nm pitch. Microcontact printing was then used to functionalize the surface of the topography with chemical patterns of fibronectin. They printed various patterns, a homogenous coating, dots, and chemical lanes that ran perpendicular to the topographical patterns. Charest *et al.* explored the combined affects of the topographical structures and chemical patterns on MC3T3-E1 osteoblast-like cells. The cells clearly preferred aligning to the fibronectin lanes over the nanogrooves. The cytoskeleton and nuclei of the cells were also visibly oriented along the fibronectin patterns.

3.2. Nanocolumns

Nanocolumns are another reoccurring motif in recent cell studies using nanopatterning techniques to create protrusions on the substrate. These designs vary from pillar-like designs to hemispherical structures using an assortment of techniques such as colloidal lithography, soft lithography, and electron beam lithography. Changes in morphology and cell attachment are typically associated with nanocolumn patterns. Using colloidal lithography, Dalby *et al.* created topographical arrangements referred to as nano-columns.^{33,34} The PMMA columns, 100 nm in diameter, 160 nm in height, and roughly 230 nm apart, were formed by using different angles of argon ion bombardment to etch the surface of the colloidal mask. The group observed smaller, rounder human fibroblasts on the nano-column surface when compared with planar control surfaces. They also saw less stress fibers developing within the cells cultured on the nano-columns. The rounder external appearance of these cells and decrease in stress fibers was likely indicative of decreased cell attachment. There was, however, increased filopodia activity from cells on the nano-column. A similar technique was also used by Dalby *et al.* to produce nanotopographical columnar structures of 144 ± 11 nm diameter, 10 ± 1 nm height, and 184 ± 24 nm pitch out of PMMA.⁵⁶ While the XY dimensions were similar to their previous study, this shorter height had a greater influence on cell adhesion, as well as differentiation. The nanostructured surface influenced human bone marrow cells taken from marrow aspirates to extend filopodia and increased cell spreading by 300% compared to the planar control. Additionally, these cells also showed an increase in expression of actin, tubulin, vimentin, and vinculin. Osteocalcin and osteopontin

production was also elevated in bone marrow cells seeded on the nanotopographical structures indicating potential osteoblast activity. Andersson *et al.* fabricated hemispherical pillars with a diameter of 167 nm and 100 nm tall on titanium and noticed a decrease in IL-6 and IL-8 production from cells on the nanotopographical patterned surfaces consisting of structures with an average height of 100 nm and average diameter of 167 nm.⁵⁷

With the use of UV curable poly(urethane acrylate) (PUA) molds, Kim *et al.* was capable of generating PEG patterns with resolutions down to 50 nm.⁵⁸ PEG-dimethacrylate (PEG-DMA) was cured with UV radiation under the PUA mold, cross-linking the PEG macromers to create customizable nanoscale structures. The group studied how P19 embryonic cells and primary heart fibroblasts interacted with PEG nanopillar patterns. The cells adhered less to the nanopillars than to unpatterned glass surfaces but more than unpatterned PEG substrates. Kim *et al.* also continued cell studies by investigating the response of primary cardiomyocytes seeded on 150 nm wide, 400 nm tall nanopillars.⁵⁹ Like earlier results, the cells cultured on nanopillars showed more adhesion compared to unpatterned PEG substrates but less than their counterparts on smooth glass substrates. The beating frequency of the cells cultured on nanopillars was significantly slower than the cells plated on glass. Collagen treated nanopillars showed a slight increase in beating frequency.

Kim et al. used holographic lithography to create a pattern of nanopits as a master stamp, and then used nanoimprint lithography to make replicas of the stamp, which could be used to generate 200 nm wide nanocolumn patterns spaced 700 nm apart.⁶⁰ Holographic lithography uses interference patterns between light waves to create consistent groove-like patterns without any masks. Human osteoblasts were cultured over the surface, and extended filopodia and lamellipodium over the pattern.

Miura and Fujimoto were able to use the Langmuir-Blodgett technique to create a colloidal monolayer of spherical 527 nm diameter poly(styrene-*co*-acrylamide) nanoparticles.⁶¹ The cells were grown on this monolayer of spherical nanoparticles instead of etching away to create nanotopographies. They seeded human umbilical vein endothelial cells (HUVEC) onto the patterned substrates, and observed pseudopods and elongated cells over the surface, which appeared to orient along the direction of the gaps between the particles. Although the cells were able to proliferate, the proliferation was higher in cells cultured on a tissue culture polystyrene dish.

3.3. Nanopits

Nanopits, another commonly studied geometry created through soft lithography techniques, have been observed to influence changes in cell morphology and adhesion. These patterns consist of holes or depressions pressed into polymer substrates using molds. Nanopit patterns with resolutions down to 30 nm in diameter have been fabricated through a combination of electron beam lithography, nickel die fabrication, and hot embossing on polymers polycaprolactone (PCL) or PMMA.^{62,63} An initial pattern was created with electron beam lithography and nickel dies were made from the pattern. The nickel die was then pressed into hot PCL to transfer the nanopattern. *h-tert* human fibroblasts and rat epitenon fibroblasts were responsive to the differences in orthogonal and hexagonal arrays of nanopits, as cells had orientations of 90° to the orthogonal pattern and 120° to the

hexagonal. There was a reduction in cell adhesion on regular arrays when compared to more irregular arrays (Figure 4).⁶³ Pits with diameters of 35 nm, 75 nm, and 120 nm pits were studied. Fibroblasts interacted extensively with 75 nm and 120 nm nanopits by extending numerous filopodia. However, the fibroblasts on the nanopits were less well-spread and had fewer stress fibers than on the control substrate. The same technique was used to create nanopits and nanopillars of 50 nm in diameter that were spaced 75, 150 or 300 nm apart.⁶⁴ The rat epitenon fibroblasts behaved similarly with reduced adhesion on both the nanopits and nanopillars, with increased adhesion as the spacing was larger. The ordered symmetry of the pattern was important, as randomly spaced dots created with colloidal lithography of similar average spacing did not decrease cell adhesion compared to planar controls.

Hart *et al.* also made orthogonal or hexagonal spaced nanopits and studied their effect on human bone marrow cells.⁶⁵ The orthogonal spaced pits had diameters of 35, 75, or 120 nm with 100, 200 or 300 nm pitch while the hexagonal was 80 nm in diameter with 300 nm spacing. It was seen that the cells were smaller and less spread on both topographies with the effects even more pronounced on the hexagonal spacing. Generating an orthogonal array of holes is also possible using electron beam lithography. Karuri *et al.* fabricated several arrays of nanoscale holes that varied from 255 nm to a little over 1000 nm in diameter with roughly a 340 nm depth and a pitch varying from 300 nm to 2000 nm. They noticed increased filopodia activity in addition to a decrease in proliferation of SV40 human corneal epithelial cells on the smallest patterns. The group noted that when the cells were exposed to fluid shear flow, the cells seeded on the smallest holes showed the greatest proliferation.¹⁹ Osteoprogenitor cells cultured on 120 nm diameter nanopits fabricated from electron beam lithography have displayed a series of positive results from interactions with the patterns. Cells cultured on the nanopits exhibited dense cell populations along with increased expression of osteopontin and osteocalcin.⁶⁶ Seunarine *et al.* created an array of nanopits with a diameter of 150 nm and center-to-center spacing of 300 nm.²⁰ These nanopatterns were then combined with a micropatterned master to make a PDMS stamp with both micro- and nano- patterned features. Here they demonstrated that cells had a different response to nanotopography when compared to microgrooves, with nanopits reducing cell adhesion.

3.4. Nanoposts/Grates

Unique nanostructures referred to as nanoposts and nanogrates have been constructed using a combination of laser interference lithography and deep reactive ion etching. The topography of these patterns possessed sharp needle- and blade-like tips (Figure 5).

The dimensions and organization of these structures are easily controllable by adjusting exposure intensities and angles. Choi *et al.* and Heydarkhan-Hagvall *et al.* made nanopost (needle-like) and nanograte (blade-like) nanostructures of 50–100 nm, 200–300 nm, and 500–600 nm in height while maintaining a distance of 230 nm between the structures. All of the posts and grates were sharpened to be needle-like.^{30,31} Human foreskin fibroblasts showed elongation and orientation to the axis of the nanopatterns. Within the cells, F-actin fibers also aligned in the direction of the nanograte structures. The elongation of the cells appeared to be influenced by the aspect ratio of the nanostructures as the cells seeded on patterns with 500–600 nm arrangements showed less adhesion. The use of laser irradiation

has also been applied to biochemical patterns. Multiphoton excitation was used to fabricate patterns of bovine serum albumin, fibrinogen, and fibronectin.³² The cross linked proteins were arranged in lines of 600 nm width and spaced 10 μ m and 40 μ m apart. Human dermal fibroblasts were observed to spread across the pitch spacing, or aligned along the patterns.

3.5. Dot/Line Arrays

Dot and line arrays are slight variations of the nanocolumn and grooved/ridged motifs, respectively, yet are predominately biochemical rather than topographical patterns. Many of the dot arrays have used block copolymer micelle lithography patterning techniques to assemble precisely patterned particles on polymer substrates. These nanoparticles can be functionalized with a variety of peptides or ligands to create highly specialized biochemical patterns targeted for specific purposes. Cell adhesion tests consisted of culturing 3T3 fibroblasts onto a pattern of 2–15 nm diameter gold nanoparticles, spaced between 15 – 200 nm apart, functionalized with cyclo(-RGDfK-) peptides patterned onto a PEG hydrogel. The cells were unable to survive or spread on the non-functionalized pattern or unpatterned PEG substrates. Cell adhesion was found to fail if the cyclo(-RGDfK-)functionalized gold particles were separated past 80 nm apart. In addition, Graeter *et al.* adapted this technique to pattern non-planar surfaces in the form of channels.³⁵ Hexagonal arrays of RGD functionalized gold nanoparticles were positioned as close as 28 nm apart. REF 52 fibroblast cells cultured on patterns with 58 nm and 108 nm spacing showed increased focal adhesion protein expression on the 58 nm pattern. Cavalcanti-Adam *et al.* speculated that the increased cell spreading observed on the 58 nm arrays was probably due to the higher density of RGD peptides present, affecting the maturation of focal adhesions.^{67,68} Each c(RGDfK)-thiol functionalized nanoparticle was 8 nm in diameter which is just wide enough for a single integrin. Arnold *et al.* was able to illustrate the importance that RGD spacing played in integrin binding as MC3T3 osteoblasts, REF 52 fibroblasts, 3T3 fibroblasts, and B16 melanocytes showed strong adhesion to the RGD functionalized 28 and 58 nm spaced patterns. Focal adhesion points and organized actin fibers were observed in cells associated with the 28 and 58 nm arrays. The cells did not adhere or spread well on functionalized arrays that were spaced farther apart than 58 nm and on non-functionalized patterns.⁶⁹ Hirschfeld-Warneken *et al.* were able to demonstrate that molecular gradients of cyclic RGDfK peptide functionalized on gold dots will affect cell adhesion and polarization of Mc3t3 osteoblasts. The gold particles formed a hexagonal pattern with 20 – 300 nm spacing that was then functionalized by the RGDfK peptide, and again demonstrated that spacing larger than 80 nm lead to limited cell adhesion, cell spreading, and little formation of focal adhesions.⁷⁰ Other recent studies have investigating cell adhesion on ordered RGD patterns created through micelle lithography have indicated that cell adhesion was strongly influenced by the spacing of the functionalized nanopatterns. These studies have observed minimum spacing dimensions required to consistently form stable focal adhesion interactions with the patterned substrate.^{71–73}

Dip pen lithography is a technique that transfers patterns onto a surface using an atomic force microscope (AFM) tip. Lee *et al.* used dip pen lithography to pattern 16-mercaptohexadecanoic acid (MHA) onto a gold surface.⁷⁴ The technique allowed dots and lines to be created as small as 100 nm in width. Retronectin, a protein that binds to integrin

receptors, was adsorbed onto the patterned MHA. 3T3 Swiss fibroblast cells were seeded onto a substrate consisting of 200 nm wide dots and 700 nm apart, and the cells were observed to attach, flatten, and elongate only on the patterned portion of the substrate.

E-beam lithography has also been employed to generate dot and line arrays of chemical, rather than topographical, patterns. Pesen *et al.* developed a novel method of producing chemical patterns using e-beam lithography.²⁴ A bovine serum albumin (BSA) covered surface was exposed to an electron beam to create a nanodot pattern. Fibronectin was then adsorbed to the exposed spots of the surface, making the fibronectin nanodots ~ 8nm tall with a radius of ~200 nm spaced 3 μ m apart. The smallest detectable nanodot was ~78 nm. Swiss 3T3 fibroblasts reacted to the fibronectin nanodots by forming focal adhesion points. Our lab has also utilized e-beam lithography to generate line arrays of collagen functionalized PEG patterns. Using a technique similar to the study by Krsko *et al.*, which produced 100 nm tall PEG hydrogels for neurite growth between the patterns, we utilized an e-beam writer to crosslink submicron amide-succinimidyI-glutarate terminated PEG patterns on indium tin oxide coated glass. After functionalizing the patterns with collagen, C2C12 skeletal myoblasts were plated on the patterned surface and extended long, thin filopodial extensions along the ~500 nm wide patterns with the same respective spacing (Figure 6).

Diblock copolymers have been utilized in alternative methods to generate nanotopographical substrates. Khor *et al.* dipped mica into a solution composed of diblock copolymers of polystyrene-block-polyvinylpyridine dissolved in chloroform to form dot-like as well as distinct worm-like nanopatterns (Figure 7).⁷⁵ The dot-like patterns were 6 nm tall, 200 nm wide, and 290 nm apart. The worm-like structures were 3.2 nm tall and 160 nm wide. They used fibroblasts and mesenchymal precursor cells to investigate the impact these patterns had on cell behavior. Fibroblasts attached and proliferated faster on worm-like patterns; whereas cells cultured on the dot-like patterns were required to send out filopodia to find anchor points and showed less adhesion and proliferation than on the plain mica and worm-like patterns. Furthermore, the study reported that the mesenchymal precursor cells exhibited thicker, more organized actin bundles on the worm-like patterns. The mesenchymal precursor cells on the worm-like structures modified their ECM and left thick layers of proteins while cells cultured on dot-like patterns did not make significant modifications to the substrate surface.

4. Summary

Recent studies have explored the use of nanoscale structures and patterns to influence cell behavior as reviewed herein. There are many different methods such as colloidal lithography, laser irradiation, electron beam lithography and stamping, which can create organized submicron and nanometer sized patterns for cell studies. A variety of patterns ranging from topographical to chemical have been shown to have an effect on cell adhesion, proliferation, alignment and gene expression, demonstrating that the nanoscale of the matrix plays a role in determining cell behavior.

The geometry of topographical patterns had a variety of effects on cell morphology. Cells grown on substrates with nanogrooves aligned to the patterns while exhibiting a variety of

cell behaviors. There was evidence of cell elongation, actin fiber orientation, changes in proliferation, increased adhesion, and increased mobility. Studies conducted using nanogrooves of varying pitches and depths concluded that deeper grooves contributed to greater cell alignment and orientation. For instance, if the grooves were less than 70 nm deep random cell spreading of fibroblasts occurred, and increased depth generally correlated with increased alignment.⁴⁹ In the XY direction, if the grooves were wider than 100 nm, the cells would not align. This may be because the size of the proteins that are implicated in contact guidance will not interact with anything so shallow or so wide, and instead ignore the nanopatterned groove. The groove depths investigated ranged from 4.4 nm to 600 nm among many studies. While increasing the depth of the groove enhanced cell alignment, it was generally associated with decreased cell adhesion. When the microscale is combined with the nanoscale, it was seen that the cell body will align to the microscale, but the filopodia interact on a nanoscale level.⁴¹

Nanopits and nanopillars also had an effect on cell shape, as cells on these types of patterns tended to be rounded up when compared to flat planar controls. For many ranges of diameters, cells were less spread out with a smaller area, but had a higher density of membrane protrusions. These membrane protrusions, or filopodia, are suggested to have a sensing role and allow cells to react to the topography. In addition, cells cultured on nanopillars or nanopits usually have reduced levels of cytoskeletal organizations, and reduced adhesion suggesting that the cells on these structures are motile and sensing rather than settled and anchored to the surface, a theory supported by several reports demonstrating increased migration on nanopillars. Finally, as the height of nanopillars increase (reported values from 160 to 400 nm tall), cells generally became smaller in area and had more disrupted cytoskeletons, and in some cases, cell viability decreased. Nanopillars and columns generally decreased cell adhesion and spreading with exception of a study performed by Dalby *et al.* in which 10 nm tall nanopillars increased cell spreading by 300%. The ordered nature of the pattern also affected the cells, as it was seen that regular and symmetrical structures would reduce cell adhesion. However, if there was random arrangement, then cell adhesion was similar to planar surfaces, suggesting that not only the size of the pattern, but the order of the topography plays an important role.

These results also seem to be cell-type dependent as one study using fibroblasts demonstrated the cells preferred to interact with the grooves and adhered less to nanopillars.^{62,76} However, axons tend to prefer to attach to the ridges as compared to the grooves.⁴² The effect on cell shape may also affect fibroblast differentiation and proliferation. As cells would need to be spread out to undergo mitosis, it was seen that the cells on the nanopillars had the least developed cytoskeletons and lower proliferation.²² These topographical effects are cell-type specific, as a similar but reduced effect was seen with endothelial cells.

Some patterns such as the colloid monolayer of nanoparticles reduced adhesion and proliferation. Many of these studies have focused on the effect of integrin spacing, with RGD-nanodots that have less than 8 nm diameter spaced at certain distances apart. RGD Au dots of less than 8 nm in size would only allow individual integrin molecules to interact, and cells could not attach when these RGD sites were blocked using an antibody.^{67,69} It was

seen that cells could not attach when RGD Au dots were spaced greater than 73 nm,^{35,67–69} and using immunostaining it was demonstrated that the vinculin was not organized and stress fibers did not form.⁶⁷ Integrins are known to play an important role with cell adhesion, and here it demonstrates that the spatial arrangement of the ECM molecules are also important.

Functionalizing nanopatterns with fibronectin or other ligands increased cell attachment and spreading. Research in this area has shown that cells prefer aligning to modified chemical patterns over topographical patterns when presented with both cues simultaneously in separate directions. Generally, cells attach to the protein regions, but can extend filopodia along the grooves. In an interesting study by Charest *et al*, it was shown that if both grooves and a chemical pattern are used, cells will usually attach to the protein lanes, unless the grooves are greater than 500 nm, in which case the cells will align along the topographical pattern (Figure 8). Ideally, a combination of biochemical cues and topographical patterns can be used to specifically direct cell behavior. A balance in topographical dimensions would then fine tune the effects the patterns have on the cells.

Nanoscale patterning holds great promise for directing cell proliferation, adhesion, morphology and gene expression. Currently the technology and tissue engineering techniques used to nanopattern chemistry or topographies is in its infancy. Patterning large areas quickly, accurately, and economically are still issues that need to be dealt with, as many of these techniques cannot be readily scaled up. Additionally, we still do not fully comprehend the biological responses to biochemical and topographical patterns. As more and more associations are made between surface interactions and internal cell behavior, increasingly complex and specific cell responses can be engineered and directed towards specific outcomes such as stem cell differentiation. This research will lead to new materials for directing and controlling cell behavior.

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Fig. 1. Nanopatterning with colloidal lithography. A and B, SEM of gold nanodots forming a hexagonal template. C, The background is passivated using PEG so that cells can only interact with the functionalized gold nanodots. D and E, fibroblasts were plated on the two patterns, and at 24 hours less cells adhered on the 110 nm pattern. (Adapted with permission from ref ³⁵)

Fig. 2. Combination micro- and nanopatterns using micro- and nanoimprint lithography. Polycarbonate nanoscale topographies were created on top of microscale patterns. MC3T3-E1 osteoblast-like cells usually aligned to the microscale patterns, yet the perpendicular nanogrooves decreased the number of cells aligned. Filopodia also interacted with the nanogrooves. (Reprinted with permission from ref ⁴¹)

Fig. 3. Nanogrooves on silicon dioxide generated with e-beam lithography. SEM images are displayed of human corneal epithelial cells cultured on nanogrooves with 70 nm ridges, 600 nm depth and 400 nm pitch (A) compared to a smooth surface (B). (Adapted with permission from ref ¹⁷)

Fig. 4. Nanopit patterns generated with hot embossing on polycaprolactone or poly(methyl methacrylate). Rat epitenon fibroblasts had reduced cell adhesion on regular arrays compared to planar services. (Adapted with permission from ref ⁶³)

Fig. 5. Needle- (nanoposts) and blade-like (nanogrates) patterns using laser interference lithography and deep reactive ion etching. A, E Controls. B, C, Nanoposts. F, G: Nanogrates. Scale bar=50 μm . (Reprinted with permission from ref ³⁰)

Fig. 6.
Phase contrast image of C2C12 myoblasts cultured on functionalized PEG nanopatterns made by e-beam crosslinking. Cells elongated along the pattern and extended thin filopodia.

Fig. 7.
Dot-like and worm-like patterns with diblock copolymers. (Adapted with permission from ref ⁷⁵)

Fig. 8. SEM image of osteoblast-like cells plated on nanogrooves overlaid with fibronectin chemical lanes. Cells preferentially aligned to fibronectin lanes but in the absence of a chemical pattern or with grooves greater than 500 nm, cells aligned to the topographical nanopattern. (Reprinted with permission from ref ⁵⁵)