Research Article

Large-Scale Protein Arrays Generated with Interferometric Lithography for Spatial Control of Cell-Material Interactions

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Understanding cellular interactions with material surfaces at the micro- and nanometer scale is essential for the development of the next generation of biomaterials. Several techniques have been used to create micro- and nanopatterned surfaces as a means of studying cellular interactions with a surface. Herein, we report the novel use of interference lithography to create a large (4 cm^2) array of 33 nm deep channels in a gold surface, to expose an antireflective coating on a silicon wafer at the bottom of the gold channels. The fabricated pores had a diameter of 140–350 nm separated by an average pitch of 304–750 nm, depending on the fabrication conditions. The gold surface was treated with 2-(2-(2-(11-mercaptopundecyloxy)ethoxy)ethoxy)ethanol to create protein-resistant areas. Fibronectin was selectively adsorbed onto the exposed antireflective coating creating nanometer-scale cell adhesive domains. A murine osteoblast cell line (MC3T3-E1) was seeded onto the surfaces and was shown to attach to the fibronectin domains and spread across the material surface.

1. Introduction

Cellular adhesion is an important process in many biological phenomena such as embryonic development, homeostasis, and pathogenesis. Although cells have micrometer dimensions, *in vivo* they are in close contact with the extracellular matrix (ECM), a substratum with topographical and chemical features of nanometer sizes [1]. Advances in material science and imaging technologies have led to the understanding that individual cell-material interactions begin with the attachment of a cell surface integrin receptor to a nano-scale peptide sequence found within ECM proteins. After adhesion of a cell, multiple integrin receptors cluster into aggregates that recruit the assembly of several cytoplasmic and intercellular proteins into a complex termed the focal adhesion. Focal adhesions are on the order of 10 nm to $10\text{ }\mu\text{m}$ and serve as crucial outside-to-inside signaling gateways that are necessary for proper cell function [2]. The micrometer- and nanometer-scale organization of surface proteins is expected to play a critical role in adhesion complex formation and function [3–7]. Seminal work in

the area of cell adhesion studies in Whitesides' laboratory patterned cell adhesive domains on the cellular scale (10s of microns), demonstrating that by controlling the shape and size of the adhesive domain, the shape and degree of physical interaction between the surface and the cell could be controlled [8–11]. Since then, cell adhesion and cellular organization has been studied extensively on micrometer-scale patterns [12–17]. However, studies of the influence of protein organization at the nanometer-scale have been limited due to a lack of a flexible, high-resolution submicron scale patterning technique that is capable of producing large enough patterned surfaces to examine a statistically relevant cell number [2].

Several techniques have been used to create surface patterns of proteins on the nanometer-scale for cellular studies [14, 17–24]. These techniques fall into four distinct categories including scanning probe, stamping, electron beam, and self-assembly [14, 17, 23]. Scanning probe techniques include nanografting, dip-pen lithography, conductive AFM, and other direct-write methods [25–30]. Stamping techniques include nanocontact printing, nanoimprint

FIGURE 1: Schematic of the interference lithography process used to create submicron protein arrays.

lithography, decal transfer, and nanomolding [31–34]. Self-assembly techniques include particle lithography, polymer-assisted templating and DNA-assisted assembly [3, 35, 36]. The above mentioned fabrication methods have been shown to be very useful to create patterns on the nano-scale, but are limited due to a high cost of fabrication or an inability to create arrays over areas large enough areas to examine statistically relevant numbers of cells [2, 23, 24]. An alternative to the methods listed above is interferometric lithography (IL), a subset of photolithography, which is capable of producing ordered nano-patterned arrays up to 4 cm² efficiently and inexpensively without the use of a mask [37–39].

IL is the process of producing an interference pattern from two or more optical beams incident from different directions. This technique may be used on a thin film of photosensitive polymer (2D, where the thickness of the photoresist is much less than wavelength of the light used) or on a thick layer (3D, where the thickness of the photoresist is much less than wavelength of the light used). The interference of two coherent beams in two-beam IL creates a standing wave that can be recorded on the photosensitive polymer over an area of 4 cm² in ~5-seconds. Figure 1 shows schematically how IL can be used to create a nano-patterned surface. The pitch, or period of the interference pattern, is governed by the following equation:

$$\text{pitch} = \frac{\lambda}{2n \sin(\theta)}, \quad (1)$$

where λ is the wavelength of the beam source, n is the index of refraction of the medium, and θ is the angle at which the two coherent beams are interfering. Features below 50 nm have been created using this technique [40]. Furthermore, the technique of IL has recently been used to create three dimensional surfaces to study the effects of micron-scale surface topography on human pulmonary fibroblast spreading and expression of inflammatory markers [41].

In this paper we describe the use of IL to produce a series of large-area (4 cm²) arrays of submicron domains that present a specific chemistry in patterned regions providing a versatile platform for the study of the effects of controlled alteration of the number, size, and spacing of focal adhesion formation during cell-material interactions. As shown in Figure 2, IL was used to pattern arrays of submicron cylindrical pillars on silicon wafers. Gold was deposited on the arrays and the polymeric pillars removed,

FIGURE 2: Schematic of the process of creating protein arrays.

creating a two-dimensional array of nano-scale domains in a gold layer. The gold surface was passivated using self-assembled monolayers (SAMs) of oligoethylene glycol (OEG) allowing for adsorption of the extracellular matrix protein fibronectin (FN) selectively to the nonpassivated array of nano-scale domains. For the studies presented here, the well-established murine osteoblast cell line MC3T3-E1 was chosen to demonstrate the availability of the adsorbed FN for cellular attachment through examination of cellular attachment and spreading on our patterned surfaces.

2. Materials and Methods

2.1. Fabrication of Nano-Patterned Structures. Silicon (Si) wafers (100) (100 mm, Siltec, Corp., Palo Alto, CA) were cleaned using an airbrush acetone spray and blow dried with N₂ gas. The wafers were then dried on a hot plate at 110°C for 2 min. A 100 nm thick layer of XHRiC-16 (Brewer Science, Inc., Rolla, MO) antireflective coating (ARC) was spun cast (5000 rpm, 30 s) and hard baked at 175°C for 3 min. Positive photoresist (PR) (SPR-505A or SPR-510A diluted in EC11 solvent (1 : 1), Shipley Company, LLC, Marlborough, MA) was spun cast (4000 rpm, 30 s) on top of the ARC followed by a soft bake at 90°C for 3 min. The coated wafers were cleaved into quarters and then exposed using an interferometric lithography corner cube arrangement with a YAG-Nd laser ($\lambda = 355$ nm, Infinity 40–100, Coherent, Inc., Santa Clara, CA) (Figure 3). In this simple arrangement, an expanded collimated laser beam is folded onto itself using a 90° geometry [37]. Cylindrical photoresist pillars were created by exposing the wafers twice for 5-seconds with the second exposure rotated 90° from the first [37, 42]. Three substrate configurations were created using different interference angles ($\theta = 36.3^\circ, 23.2^\circ, 13.7^\circ$, yielding pitches of 300 nm, 450 nm, and 750 nm, resp.). After exposure, each sample was soft baked at 110°C for 1 min and the developer (undiluted MF702 developer, Shipley Co.) was

FIGURE 3: Corner cube IL arrangement used to create large-area arrays.

added dropwise to form a puddle on the surface over 45 s. Finally the samples were rinsed with deionized water and blown dry with N_2 gas.

The developed chips were placed in an e-beam evaporator (Consolidated Vacuum Corp., Rochester, NY) where a 3 nm layer of Cr was deposited on top of the patterned substrate followed by the deposition of 30 nm of gold (99.999%, Plasmaterials, Inc., Livermore, CA). After metal evaporation, the remaining photoresist pillars were lifted off by sonicating the Cr/gold-covered chips in acetone for 30 min, leaving an array of nanometer-scale ARC patches in the gold covered surface.

2.2. Nanometer-Scale Protein Adhesive Domains. The patterned chips were further cleaved into 1 cm^2 squares. The gold covered surface was functionalized by incubating the substrate in a 1 mM solution of $\text{HS}(\text{CH}_2)_{11}(\text{OCH}_2\text{CH}_2)_3\text{OH}$, 2-(2-(2-(11-mercaptopundecyloxy)ethoxy)ethoxy)ethanol, (OEG, 95%, Sigma-Aldrich, St. Louis, MO) in ethanol for 24 hrs. To create protein-adhesive domains, fibronectin (FN) from bovine plasma was adsorbed onto the ARC patches by incubating a FN solution ($5\text{ }\mu\text{g/mL}$, in Dulbecco's Phosphate Buffered Saline (PBS), GIBCO, Invitrogen Corp., Carlsbad, CA) for 1 hr at 4°C on an orbital shaker.

2.3. Microscopy. Topography of the substrates with bound OEG was imaged with a scanning probe microscope/atomic force microscope (tip) (Digital Instruments CP-II, Veeco, Plainview, New York) equipped with a Veeco Si tip in tapping mode. After thiol adsorption the samples were air dried and mounted on an aluminum holder for AFM scanning. Images of the pillared and the gold coated surfaces were taken using a field-emission scanning electron microscope (SEM) (Hitachi S5200 Nano SEM, Pleasanton, CA). To image cell spreading using SEM, substrates with adherent cells were prepared by fixing in 2.5% glutaraldehyde for 45 min at room temperature and then dehydrated with increasing amounts of ethyl alcohol (30%–100%, 5 min each), rinsing with PBS after each ethanol step.

2.4. Characterization of Protein-Adhesive Domains. Gold labeled antibodies (Nanogold conjugates, Nanoprobes, Inc., Stony Brook, NY) were used to visualize proteins bound to

the patterned substrates. After FN adsorption, the surfaces were rinsed with PBS to wash off any weakly adsorbed FN. The substrates were incubated in a solution of antiFN primary antibodies in PBS (1 : 400, AB2047, Millipore Corp., Billerica, MA) for 1 hr at 4°C on an orbital shaker and washed with PBS after antibody binding. Secondary gold-labeled antibodies in PBS (1 : 400) were incubated with the substrates to allow for binding to the primary antibody following the same procedure as the described above. GoldEnhance EM (Nanoprobes Inc.) was applied for 5 min following the manufacturer's recommended protocol to increase the size of the gold-labeled antibodies for better visualization with electron microscopy.

2.5. Cell Culture. The murine osteoblast cell line MC3T3-E1 (CRL-2593, ATCC, Manassas, VA) was used to demonstrate accessibility of the adsorbed FN for cellular attachment through attachment, viability, and spreading studies. These cells were chosen as they are a well-established, widely used cell line. Before seeding, cells were maintained in α -Minimum Essential Medium (α -MEM, GIBCO, Invitrogen Corp.) supplemented with 10% fetal bovine serum (FBS, GIBCO, Invitrogen Corp.) and 1% penicillin-streptomycin (GIBCO, Invitrogen Corp.) and passaged every 3 days using standard techniques. To passage, adherent cells were rinsed with PBS, enzymatically lifted through incubation with 0.25% trypsin-2,2',2'',2'''-(ethane-1,2diylidinitrilo)tetraacetic acid (trypsin-EDTA, Fisher Scientific, Waltham, MA) for 10 min, and equally divided among three new flasks (T-75, Fisher Scientific). Prior to cell seeding, cells were enzymatically lifted and counted with a hemocytometer. The cells were seeded onto the surfaces at a density of 2.5×10^4 cells/ cm^2 and incubated at 3°C and 5% CO_2 in a humidified environment.

2.6. Cell Studies. To determine cellular attachment affinity for each of the array component materials, cells were seeded on flat, nonpatterned surfaces of ARC after incubation in a fibronectin solution as described for the patterned surfaces (ARC + FN), ARC without incubation prior to cell seeding (ARC), OEG SAM functionalized ARC incubated in a FN solution (OEG + FN), and OEG alone (OEG). Glass microscope slides without incubation in a FN solution was used as a positive control (Glass). After 4 hrs of incubation, cell-seeded substrates were rinsed twice with PBS to remove any unattached cells. Cell number was determined using the CeliTitre-Glo Luminescent Cell Viability Assay Kit (G7S71, Promega, San Luis Obispo, CA). This assay quantitates the amount of ATP released from the adherent cells after incubation in a cell lysis buffer. The measured luminescence is then compared to a standard curve created with known numbers of cells.

On the array surfaces, a LIVE/DEAD Viability/Cytotoxicity Assay Kit (L-3224, Invitrogen, Carlsbad, CA) was used according to manufacturer's instructions to image and assess cellular viability on the patterned substrates. For this controlled study, ARC surfaces were used as the positive control. After 4 hrs of culture, cells were rinsed with PBS

twice. Cells were then treated with a calcein acetoxymethyl ester:ethidium homodimer-1 solution ($2\mu\text{M}:4\mu\text{M}$) and incubated for 30 min in the dark at room temperature. Ubiquitous presence of esterase activity on live cells converts calcein AM into green fluorescent calcein. Ethidium homodimer-1 penetrates damaged membranes of dead cells and fluoresces red as it binds nucleic acids. Following incubation, cells were rinsed with PBS three times and fixed in 4% formaldehyde in PBS for 10 min. The substrates were mounted on a microscope slide using antifade mounting solution (5013, Millipore Corp., Billerica, MA) and the entire surface was imaged using a confocal microscope (Zeiss LSM 510-META, Thornwood, NY). Cell counts of live and dead cells for three samples were used for viability calculations.

2.7. Statistical Analysis. Results were analyzed using the Kruskal-Wallis one-way nonparametric analysis of variance (ANOVA) with values of $p < 0.05$ considered statistically significant.

3. Results and Discussion

3.1. Fabrication of Arrays of SubMicron Protein Domains. Silicon wafers were treated initially with a layer of ARC to prevent reflection from the Si substrate back onto the photoresist. Positive photoresists formulations SPR505A and SPR510A were spun onto the ARC-treated Si wafers. A corner-cube arrangement was used to create 2D square arrays (Figure 3). The square array was obtained by exposing the photoresist twice for 5-seconds, with the second exposure at 90° from the first. The resulting structure is a 2D array of cylindrical posts with diameter and pitch determined by the angle of interference, θ [37, 42]. Following exposure and development, the cylindrical posts on the ARC were visualized using SEM. Figure 4 shows post arrays for interference angles of 36.3° , 23.2° , and 13.7° created with an ultraviolet beam source ($\lambda = 355\text{ nm}$) in air ($n = 1$). Figure 4(a) shows an SEM micrograph of posts with a diameter close to 140 nm and a pitch of 300 nm, created by an angle of interference of 36.3° . As the angle of interference was reduced to 23.2° and then 13.7° the pitch increased from 450 nm to 750 nm, respectively (Figures 4(b) and 4(c)). With our interference lithography setup, as the pitch increased, the post diameter also increased, with a pitch-to-post ratio remaining at $\sim 2 : 1$.

A 3 nm chromium adhesion layer followed by 30 nm layer of gold was deposited on the surfaces of the patterned substrates. Acetone was used to selectively remove the cylindrical posts, exposing submicron sized ARC patches in a gold field. Figure 5 presents scanning electron micrograph images of surfaces after removal of the photoresist posts for the three interference angles examined, 36.3° (Figure 5(a)), 23.2° (Figure 5(b)), and 13.7° (Figure 5(c)). All three images show the presence of ordered arrays in the gold surface with complete removal of the photoresist pillars. Scanning electron micrographs from three different samples for each pitch size were used to determine and average the pitch distance and patch diameter, with ten random pitches/patches

(a)

(b)

(c)

FIGURE 4: SEM micrographs of 2D arrays of posts. (a) 300 nm pitch ($\theta = 36.3^\circ$). (b) 450 nm pitch ($\theta = 23.2^\circ$). (c) 750 nm pitch ($\theta = 13.7^\circ$). Photoresist posts are created after a 2D exposure of two-beam IL.

measured per sample. From these measurements, individual patch size and total cell adhesive area per square micron were calculated (Table 1). While the number of domains per square micron decreased with increasing patch diameter and pitch, the total area of all cell adhesive domains per square micron did not change significantly (ANOVA $p = 0.096$). Because of this, these surfaces are ideal for isolating the effects of variations in the number, size, and spacing of attachment sites a cell is exposed to on a surface from changes in the total attachment area.

To passivate the background, the gold field was treated with an ethanolic solution of 2-(2-(2-(11-mercaptoundecyloxy)ethoxy)ethoxy)ethanol (OEG). It has been previously shown that self-assembled monolayers (SAMs) of oligoethylene glycol terminated thiols assembled on gold surfaces do not allow protein binding. Because cells require the presence of proteins in their correct conformation in order to attach to a surface, the OEG SAMs do not allow for or support cellular attachment [9, 43, 44]. The submicron-scale domains on the surfaces as well as the homogeneity of the OEG-SAM layer was visualized with AFM. Figure 6 shows a representative AFM image of a $4\mu\text{m}^2$ area of a surface with a pitch of 300 nm ($\theta = 36.3^\circ$, patch diameter of 150 nm) after SAM formation. Because the SAMs bound selectively on the gold surface and not the noncoated submicron domains, the domains can be distinguished on sample as regularly ordered depressions in SAM surface. During fabrication, 37 nm thick layer of metal was deposited on the surfaces. The AFM image shows a consistent layer of material approximately 40 nm tall in all areas except the circular domains, which is expected as a OEG SAM layer is approximately 2 nm thick [45].

To render the ARC patches cell adhesive, the patterned substrates were incubated with FN, an extracellular matrix protein known to bind with integrins expressed by the murine osteoblast cell line MC3T3-E1 [46]. Scanning electron microscope images were used to locate FN on the surfaces after incubation with the protein solution. To allow for clearer imaging of the adsorbed proteins and ensure differentiation between the FN and other surface features, samples were exposed to a solution of primary antibodies specific to FN followed by labeling of the primary with secondary antibodies labeled with gold nanoparticles. The gold label was enhanced with the use of the gold deposition solution GoldEnhance EM (Nanoprobes, Inc.). Figure 7 shows the enhanced gold-labeled antibodies, indicating the presence of FN, located at the ARC domains and not throughout the OEG SAM surface. In the SEM images the protein appears to be localized at the edges of the ARC domains. This attachment pattern within the ARC domain may be an artifact of the dehydration process the samples must undergo to allow visualization via SEM. As the aqueous phase evaporates out of the pores created by the SAMs, the protein is forced the edges and remains there upon complete dehydration.

3.2. Cellular Attachment to Individual Material Components. Before seeding cells on the arrays, studies were performed to examine the attachment affinity of the cells to each of the material components of the patterned substrates. Figure 8 shows the results of attachment studies of the murine osteoblast cell line MC3T3-E1 on nonpatterned ARC surfaces after incubation with FN, ARC alone, OEG SAMs after incubation with FN, OEG SAMs alone, and glass substrates. Cellular attachment was statistically greater on ARC surfaces after incubation with FN than any other treatment group examined. The OEG SAMs exhibited minimal cell attachment regardless of incubation with

FIGURE 5: SEM micrographs of resulting patterned submicron-sized ARC patches after photoresist lift-off. (a) 300 nm pitch. (b) 450 nm pitch. (c) 750 nm pitch.

FIGURE 6: AFM scan of a representative area ($4\mu\text{m}^2$) of a 100 nm domain array after OEG adsorption. The metal/SAM layer is found to be approximately 40 nm above the ARC surface as expected. The SAM surface is a consistent height, showing a lack of defects.

TABLE 1: Patch diameter related to the pitch distance.

Angle of interference	Theoretical pitch (nm)	Theoretical domain diameter (nm)	Measured pitch (nm)	Measured domain diameter (nm)	Area per domain $\times 10^{-2} (\mu\text{m}^2)$	Number of Domains per μm^2	Total domain area per μm^2 ($\times 10^{-2} (\mu\text{m}^2)$)
36.3°	300	150	304 $\pm$ 8	140 $\pm$ 10	1.5 $\pm$ 0.2	9	14 $\pm$ 1
23.2°	450	225	460 $\pm$ 20	200 $\pm$ 30	3.1 $\pm$ 0.6	4	13 $\pm$ 3
13.7°	750	375	750 $\pm$ 20	350 $\pm$ 20	9.6 $\pm$ 0.8	1	10 $\pm$ 1

FIGURE 7: SEM micrograph of 100 nm domain array after SAM formation and FN adsorption. Gold nanoparticle-labeled antibodies and a gold enhance solution was used to visualize location of FN on the surface. Note that the FN has selectively adsorbed to the submicron domains and cannot be found throughout the SAM surface.

FIGURE 8: Cellular attachment on protein array component materials (positive control: glass) (* $p < 0.05$, *** $p < 0.001$).

fibronectin. These results were expected as previous studies have shown OEG SAMs fabricated with 2-(2-(2-(11-mercaptopundecyloxy)ethoxy)ethoxy)ethanol resist protein adsorption due to tight packing of the OEG chains with minimal defects [9]. Without proteins with which to interact on the OEG SAM surface, cells cannot attach. These results suggest that cells will adhere selectively to the protein domains of our arrays. The increased cellular attachment to the ARC surface post-attachment compared to either of the OEG surfaces is likely due to nonspecific adsorption of the proteins present in the cell culture media.

3.3. Availability of Adsorbed FN for Cell Attachment on Protein Arrays. To determine whether the FN of the adsorbed protein array was accessible to cells, arrays were seeded with a murine osteoblast cell line. These cells were chosen as a model cell line to verify cellular attachment and viability on our arrays as they are well characterized and their behavior well studied. The cells were allowed to attach and spread on 1 cm² arrays over 12 hrs followed by fluorescent staining with a LIVE/DEAD Viability/Cytotoxicity Assay. This assay stains live cells green and dead cells red. Confocal images of the stained cells on arrays and as well as nonpatterned ARC-coated Si wafers after incubation with FN are presented in Figures 9(a) and 9(b), respectively. The flat ARC-coated FN surfaces were used as positive controls in order to determine if the patterned surfaces did not allow for cellular attachment. Both surfaces show good attachment and spreading with no evidence of post-attachment cell death. Quantification of the cell numbers showed no differences in cell number between the pattern substrates and the positive controls (data not shown).

While cellular attachment and spreading could be visualized with a fluorescent microscope, scanning electron microscopy was necessary to directly examine the cell/material interactions. Scanning electron microscopy allows for the imaging of the cells and material surface features directly at a much greater magnification than traditional fluorescent microscopy. Figure 10 presents representative images of cells on the arrays 4 hrs after seeding. As seen in the fluorescent images, the cells have attached and spread on the arrays. Filopodia are seen spreading out from the cell bodies, following paths through the protein arrays (Figures 10(a) and 10(b)). At higher magnifications, the cells can be seen probing and exploring the submicron domains (Figures 10(b) and 10(d)).

4. Conclusions

Interference lithography was implemented successfully to create large-scale protein arrays. IL is an attractive alternative to many existing techniques as it can create ordered structures without the need for a phase mask or stamp and can create arrays up to 4 cm² with just two 5-second exposure steps. IL was used to create arrays of submicron diameter pillars on ARC-coated silicon wafers. These surfaces were further treated to create arrays of adsorbed FN in a passivated background. Murine osteoblasts (MC3T3-E1) were used to demonstrate the availability of FN for cellular interactions through cellular attachment and spreading studies on the protein arrays. Just four hours after seeding, cells were

FIGURE 9: Fluorescent images of MC3T3 cells 12 hrs after seeding on (a) positive control ARC-coated Si wafer with adsorbed FN and (b) 100 nm domain protein array. Using the Live/Dead Viability Assay, live cells appear green whereas dead cells appear red.

FIGURE 10: Representative scanning electron micrographs of MC3T3 cells on protein arrays. (a) and (b) show cell attachment and spreading; (c) and (d) demonstrate extension of cellular processes as cells probe the surface and selectively attach to protein domains.

shown to be extending projections, connecting the cell to the adhesive domains on the patterned surfaces. These patterned cell adhesive domain arrays offer a cell culture platform to study the effects of systematic manipulation of size and distribution of cell/material interaction points on cellular processes.

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