Monitoring of elongation and orientation of osteoblast cells directed by anisotropic nanopatterns

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Introduction

Nanoscale surface topology of extracellular matrix (ECM) is recognized as one of the most important environmental cues for initial cell guidance.[1,2] Earlier reports have shown that cell responses, including morphology, orientation, and migration, can affect gene expression and even the fate of cells. On the other hand, studies on cell response to nanoscale topology are lagging behind due to the complexity of cell behavior and the difficulties in fabricating materials with controlled nanoscale topology.

In this study, we monitored the time dependence of cell alignment and morphology (i.e., elongation) on anisotropic nanoscale gratings. Previous results have indicated that orientation of osteoblast cells can be directed by anisotropic patterns or gratings, but results were often collected using fixed cells and on patterns with dimensions in the micron length scale. Herein, we investigated the behavior of pre-osteoblasts cultured on nanoscale gradient patterns of transparent photopolymerized dimethacrylates. These patterns had a 200 nm or 400 nm line width and a corresponding 400 nm or 800 nm pitch. The pattern height decreased from several hundred nanometers on one end of the substrate to a flat surface on the other end of the substrate in continuous fashion. The morphology change of the cells on these patterns was studied, and cell alignment along the pattern and cell elongation as a function of pattern height were examined as a function of incubation time.

Methods[†]

Photo-activation of the Dimethacrylate Resin. Ethoxylated bisphenol-A dimethacrylate (degree of ethoxylation = 6, Esstech Inc.) was activated using the photoinitiator system camphorquinone (0.2 % by mass, Aldrich) and ethyl 4-N,N-dimethylaminobenzoate (0.8 % by mass, Aldrich) and stored in the dark until use.

Nanoimprinting. The imprints were made into poly(styrene) (PS) with a molecular mass of 18.7 kg/mol (Polymer Laboratories). PS was dissolved in toluene at a concentration of 6.7 % by mass. The films were spun cast at 209 rad/s (2000 rpm) onto Si wafers with a native oxide surface. The spun cast films were baked under vacuum for 30 min at 170 °C to remove the residual solvent before imprinting. The imprints were made on a NX-2000 imprint tool with a mold consisting of parallel line-and-space gratings in silicon oxide with a pitch of 400 nm or 800 nm and a pattern height of 360 nm. The mold surface was treated with a low-energy self-assembled monolayer deposited from tridecafluoro-1,1,2,2 (tetrahydroctyl)trichlorsilane vapor to facilitate mold release. After sufficiently evacuating the sample chamber, the imprints were made in two steps: 10 s at 100 °C and 1.4 MPa followed by 3 min at 140 °C and 3.4 MPa. The imprint tool was then cooled to 55 °C before releasing the pressure and separating the pattern from the mold. To form the gradient in pattern height, imprinted structures were annealed on a hot stage with a range of temperatures for a predefined period of time. The imprinted PS substrates were used as a template to produce photopolymerized dimethacrylate based gradient structures.

Atomic Force Microscopy. Atomic force microscopy (AFM, Dimension 3100, Vecco Instruments) was used to image the patterns. Two sets of experiments were carried out to verify the reproducibility of these AFM experiments. In this study, the purpose of AFM analysis was limited to extracting the pattern height, not the full pattern profile. Special care was taken to ensure that AFM tips always touched the bottom of the trench between two adjacent lines to obtain an accurate measurement of the pattern height. The height measurements were calibrated with standard grating patterns with line height 400 nm. For 400 nm and 800 nm pitch patterns, 3 µm x 3 µm and 5 µm x 5 µm scans were performed, respectively. Uniformity of the pattern shape/height was confirmed by performing measurements at multiple spots on each sample.

Cell Seeding and Live Imaging. The MC3T3-E1 subclone 4 murine pre-osteoblast cell line was purchased from the American Type Culture Collection. Nanopatterned PS samples were sterilized in 70 % (by volume) ethanol for 20 min. Cells were seeded on nanopatterns at densities of 10 x 10^3 cells/cm^2. A time series of live, phase contrast images of the cells on the patterns was collected using an inverted Nikon microscope. Images were collected 20 min, 2 hr, and 24 hr after seeding. Fiduciary markers were used to locate the same positions at each time point. Cells were returned to the 37 °C incubator in between images.

Cell Elongation and Alignment. After 48 h of culture, samples were fixed with 3.7 % (by volume) formaldehyde, permeabilized with 0.5 % (by volume) Triton X-100, blocked with 10 mg/mL bovine serum albumin, rinsed with PBS, and stained for 1 h. Cell nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI, Invitrogen, 1:10000); cell bodies were stained with Alexa Fluor 488 C_5-maleimide (Invitrogen, 1:5000); F-actin was stained with Alexa Fluor 546-Phalloidin (Invitrogen (1:40). Cell images were captured using a Leica DMR 1200 upright microscope, a 10X objective, and a Hamamatsu Orca ER digital camera (Vashaw Scientific, Inc.). Color composites of the cell components were created using ImagePro Plus software (Media Cybernetics). Elongation and alignment of cell bodies were characterized using ImagePro Plus. The elongation values evaluated the extension of the cells using the ratio of the long axis of the cell to the short axis of the cell, and the alignment described the cell orientation with respect to the grating direction. Approximately 100 cells were analyzed for each position.

Results and Discussion

Annealing of an imprinted structure above the glass transition temperature (T_g) produced a gradient in pattern height. This gradient pattern was successfully onto a dimethacrylate material, which produced an optically clear, free-standing film. The precise height profile and the surface morphology of the gradient patterns were examined using AFM (example shown in Figure 1). The height profile depended on the annealing conditions.

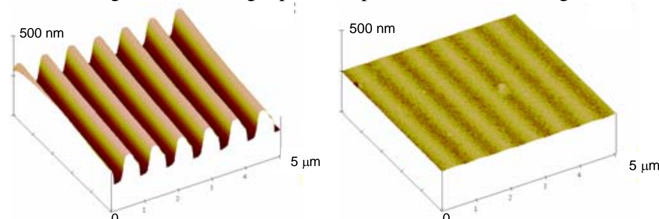

Figure 1. AFM height images for dimethacrylate grating patterns. Images are shown for a high pattern (left) and a low pattern (right).

Osteoblast cells were found to respond to the surface topology, particularly at high patterns. The cell responses include cell alignment and elongation, among other characteristics. At 48 h, cells were well-aligned along the axis of the grating on structures with large pattern height, but the orientation became more random as the height decreased (Fig. 2). Aligned cell also exhibited stretched F-actin fibers along the long axis of the cells. In most cases, the nuclei were also aligned to the long axis of the cells.

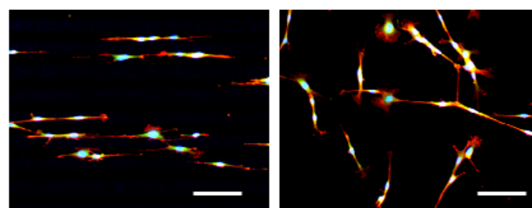

Figure 2. Epifluorescence microscope images of cells cultured for 48 h on the dimethacrylate patterns. Cells were well aligned with the grating at high patterns (left) and poorly aligned at low patterns (right). Scale bar = 100 µm.

[†] Certain equipment, instruments or materials are identified in this paper in order to adequately specify the experimental details. Such identification does not imply recommendation by the National Institute of Standards and Technology nor does it imply the materials are necessarily the best available for the purpose.

To better characterize the cell alignment and elongation, a time series of live cell images was collected to determine the kinetics of alignment. One advantage of our dimethacrylate gradients is their transparency. The cells are easily imaged using a standard inverted microscope and phase contrast objectives. Live imaging of cells cultured on grating patterns fixed to silicon wafers require additional equipment and most likely fluorescent staining of the cells, which may interfere with regular cell processes.

Interestingly, images of the cells revealed that cell alignment began shortly after cell seeding. For instance, cells imaged 20 min and 120 min after seeding showed clear cell alignment early (within 20 min) in the cell attachment process (Fig. 3). The cells then proceeded to elongate along the pattern by extending cellular processes, as seen at 120 min.

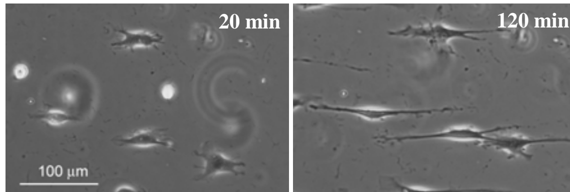

Figure 3. Live imaging of cells on the high end of the dimethacrylate gradient pattern. The cells began to align with the grating pattern as early as 20 min after cell seeding (left). Cell elongation was evident at later time points, such as 120 min after seeding (right). Both images were collected at the same location on the same gradient sample.

Conclusions

The gradient patterns created first in polystyrene were successfully duplicated onto the dimethacrylate polymers. The transparent, dimethacrylate based patterns then served as a platform to investigate the alignment and elongation of cells via live cell imaging as a function of culturing time and pattern height. These patterns with nanoscale gradient topology are an efficient way to observe the initial response of the cells to the grating pattern. Osteoblast cells showed significant differences in alignment as a function of the pattern height. By analyzing the orientation of the cells, the critical height for cell alignment can be determined. The transparent patterns also showed potential as a convenient and nondestructive tool to monitor cells in situ. These patterned materials show great potential for developing medical devices with nanoscale features and studying tissue engineering with varying nano-topographies.

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