Monitoring Alignment of Osteoblast Cells Directed by Gradient Nanopatterns

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INTRODUCTION

Cells respond to topographical structures on multiple length scales from macroscopic features down to the molecular levels.[1] The majority of studies on the interactions between cells and the surface landscape, including features such as grooves, ridges, and wells, have been carried out in the micrometer range. Recent studies have indicated that cell behavior can be modified using artificial nanoscale features with sizes and shapes similar to important cellular functional components or extracellular matrix structures, such as actin fibers, basement membrane proteins (10 to 300 nm in diameter), interconnecting nanopores, and hydroxyapatite crystals (4 nm).[2] In order to design advanced medical devices with precise control of cellular behavior, understanding the cell response to nanometer scale surroundings is crucial.

The size scale of topographical features is decreasing, approaching tens of nanometers, but detailed cell/substrate interactions for surfaces with nanoscale features are still lacking, partly due to difficulty in fabricating biocompatible materials with well-controlled nanoscale topographical features.[3-5] A single surface that includes topographical features encompassing a range of length scales will help abridge this challenge and expedite research and development in this field.

In this study, polystyrene surfaces with gradient patterns varying in height from ~360 nm to 0 nm were prepared using nanoimprinting technology and a temperature-controlled stage. We investigated the behavior of pre-osteoblasts cultured on gradient patterns imprinted on polystyrene and photopolymerized ethoxylated bisphenol-A dimethacrylate substrates. These patterns consisted of a 200 or 400 nm line width and a 400 or 800 nm pitch. The pattern height varied in a continuous fashion from several hundred nanometers to a flat surface. The morphology 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.

METHODS[†]

Reagents. Ethoxylated bisphenol-A dimethacrylate, was obtained from Esstech Inc. The photoinitiator system of camphorquinone (CQ) and ethyl 4-N,N-dimethylaminobenzoate (4E) was purchased from Aldrich Corp. All reagents were used as received. The resin was activated with a redox photoinitiator system consisting of 0.2 % CQ and 0.8 % 4E (by mass) and stored in the dark until use.

Nanoimprinting. The imprints were made into poly(styrene) (PS) standard 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 grating in silicon oxide with pitch of 400 nm and 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 patterns, imprinted structures were annealed on a hot stage with a range of temperatures for a predefined period of time.[6] The imprinted PS substrates were used to culture cells directly or as a template to produce dimethacrylate based gradient structures.

Cell Seeding and Staining. 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 of passage 4 to 10 were seeded on the nanopatterns at densities of 10 x 10^3 cells/cm^2. After 48 h of culture, the samples were fixed using 3.7 % (by volume) formaldehyde in phosphate buffered saline (PBS) for 20 min. The cells were then permeabilized with 0.5 % (by volume) Triton X-100 for 15 min, blocked with 10 mg/mL bovine serum albumin in PBS, and rinsed with PBS. Cell nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI, Invitrogen) at a 1:10000 dilution in PBS for 1 h; cell bodies were stained with Alexa Fluor 488 C_5-maleimide (Invitrogen) at 1:5000 dilution in PBS for 1h; and F-actin was stained for 1 h with Alexa Fluor 546-Phalloidin (Invitrogen) diluted 40-fold in PBS. Samples were rinsed and imaged in PBS.

Confocal Microscopy. Laser scanning confocal microscopy was carried out using a Zeiss LSM 510 confocal microscope with 1 Airy-disk-unit pinholes in reflectance mode. A water immersion objective (10X magnification) was used to collect tile images of the entire nanopatterned sample. The cell body stain was visualized using the 488 nm excitation laser and a bandpass emission filter (505 nm to 550 nm).

Epifluorescence Microscopy. Images of cells on the polymer films were also captured using a Leica DMR 1200 upright microscope, a 10X objective, and a Hamamatsu Orca ER digital camera (Vashaw Scientific, Inc.). Each field of view was imaged using three different microscope settings: epifluorescence with a blue filter cube for cell nuclei (DAPI), epifluorescence with a red filter cube for F-actin, and epifluorescence with a green filter cube for cell bodies. Color composites were created by combining the three images using Image Pro software (Media Cybernetics).

Elongation and Alignment Characterization. Images from five separate regions varying in structure height were selected from the tile images obtained using the confocal microscope. The elongation and alignment of the cell bodies were characterized using Image Pro software. The elongation values evaluated the aspect ratio of the cells, and the alignment described cell orientation with respect to the grating.

RESULTS AND DISCUSSION

Annealing the PS imprinted structure above the T_g produced a gradient in the pattern height. The precise height profile was controlled by the temperature range and the annealing time. The surface morphology of the gradient patterns was examined using AFM (example shown in Figure 1). The original imprinted structure had a pitch of 400 or 800 nm and a height of 360 nm. The height decreased non-linearly along the substrate due to slumping of the imprinted structures annealed above the T_g.

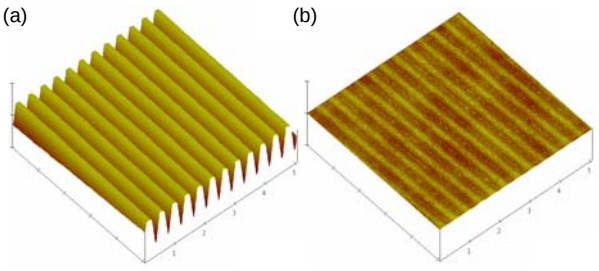

Figure 1. AFM height images for PS grating patterns (a) before slumping (b) after slumping (x and y scale: 5 μm, z scale: 0.4 μm).

The cell morphology, including alignment and elongation changed as the pattern height changed. Cells were well-aligned on imprinted

[†] 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.

structures with 360 nm pattern height, and exhibited preferential alignment on structures with sufficient height. The cell orientation became random as the height decreased (Figure 2). The cells aligned along the axis of the grating, and the F-actin fibers were stretched along the long axis of the cells. In most cases, the nuclei were also aligned to the long axis of the cells.

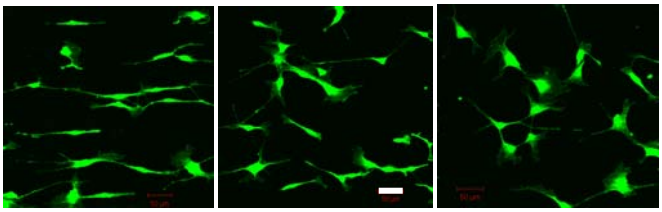

Figure 2. Cells on photopolymerized dimethacrylate gradient patterns. The cells were well-aligned on the imprinted structures (left image), somewhat aligned as the imprint structure height decreases due to annealing (middle image), and randomly oriented on the nearly flat surface (right image). The cell bodies were labeled with Alexa Fluor 488 C_5-maleimide (green). Scale bar = 50 μm.

Elongation of cells was calculated as the ratio of the long axis of the cell to the axis perpendicular to the long axis. The elongation factor (E) was defined as this ratio minus one. The E factor was much greater for cells on the patterned surfaces than on the flat surfaces.

Similar cell morphologies were observed on both PS and dimethacrylate surfaces, but the dimethacrylates were optically clear, free-standing films. Culturing the cells on transparent patterns enabled us to monitor the cell morphology on the live cells. Cell alignment occurred shortly after cell attachment, and the cells remained aligned.

CONCLUSIONS

In this study, the gradient patterns served as a platform to investigate the alignment and elongation of cells on gratings with varying height and pitch as small as 400 nm. This gradient nanopattern is an economical and efficient way to observe cells at different nanotopographies. The osteoblasts showed significant differences in alignment and elongation 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. The nanoimprinting technology as well as these patterned materials show great potential for developing medical devices with nanoscale features and for engineering tissues with varying nanotopographies.

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