CELL ADHESIONS ON NANOTURF SURFACES

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ABSTRACT

We report on various aspects of cell adhesion of fibrolasts over densely-populated sharp-tip nano-post structures, which we term "NanoTurf". The ability to control the size, shape, and aspect ratio of the nanostructures enabled the study on the effect of surface three-dimensionality of the cell-matrix adhesion in detail. To our best knowledge, this is the first systematic investigation of the nanometric three-dimensional surface topography effect on cell adhesions.

1. INTRODUCTION

Cell-matrix adhesion *in vivo* is a three-dimensional (3D) nano-environmental phenomenon. 3D adhesion differs from focal and fibrillar adhesions characterized on two-dimensional (2D) substrates *in vitro* [1-3]. The full complement of matrix topography, molecular composition, and mechanical properties (e.g., pliability) are reportedly important for adhesions to form properly and for activation of desired intracellular pathways [1, 2]. However, the specific contribution of matrix topography to the formation of cell adhesions is still poorly understood. Although several cell behaviors over various surface topographies have been studied with micro- and nano-structured surfaces [4-6], there is relatively little information on the reactions to the nano-structured surfaces. The effect of nano-periodic surface features on cell behaviors have been examined using several nanoscale structures such as gratings [7-9], columns [10-12], dots [13, 14], pits [15], and meshwork [16] created by various nanolithography and nanofabrication techniques including laser holography [7], laser irradiation [9], electron-beam lithography [8, 15], black silicon method [10], polymer demixing [11], colloidal lithography [12], dip-pen lithography [13], block-copolymer lithography [14], and carbon nanotubes [16]. However the serial methods [8, 9, 13, 15] are not proper to pattern a large area effectively, and the patterns from the parallel methods [10-12, 14, 16] are poorly ordered or random in their structures. The ability to control the surface topography, especially in the nanoscale, was very limited such that it was difficult to isolate the effect of three-dimensionality of nanoscale surface features on cell adhesions.

In this paper, we describe the post structures of silicon NanoTurf, whose size, tip shape, and height can be independently controlled to represent various three-dimensionalities of a surface and to differentiate the 3D nano-topographical effect on the cell adhesions. We report our observations on fibroblast attachment, viability, morphology, proliferation, extension, and extracellular deposition over these 3D nano-environments. In particular, the effect of the height (or aspect ratio) of the nano-post structures having very sharp tips is examined in this report.

2. SAMPLE PREPARATION

Nanostructure Fabrication

A simple but effective method to fabricate silicon nanostructures over a large area with superior control of pattern regularity has been reported by our group, using interference lithography and deep reactive ion etching (DRIE) [17]. It was shown that the sidewall profile of nanostructures could be regulated and even programmed as desired and the tips can be sharpened. Figure 1(a) shows the atomic force microscopy (AFM) scan of the photoresist (PR) pattern created by the interference lithography, which is directly used as an etch mask in DRIE. Figures 1(b)-(d) show three different aspect ratios of silicon nanostructures with a pitch (i.e., period) of ~230 nm. The tips were all sharpened.

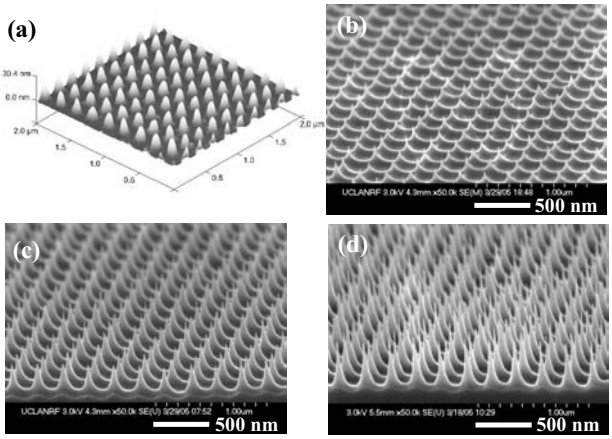

Figure 1: (a) AFM scan of PR pattern created by interference lithography for NanoTurf fabrication. The height of the PR pattern is ~20 nm. (b)-(d) Scanning electron microscopy (SEM) pictures of ~230 nm pitch (i.e., period) nano-posts: (b) Low aspect ratio (~100 nm in height), (c) Middle aspect ratio (~250 nm in height), (d) High aspect ratio (~400 nm in height).

Cell Culture

The procedure of cardiac fibroblasts isolation consisted of five enzymatic digestions (0.02% collagenase (Worthington) and 0.06% pancreatin (Sigma)) of the ventricles from 1- to 2-day-old neonatal Dawley rats. This protocol followed in order to increase the fraction of fibroblasts/cardiac myocytes. The isolated cells were re-suspended in Dulbecco´s Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen), penicillin/

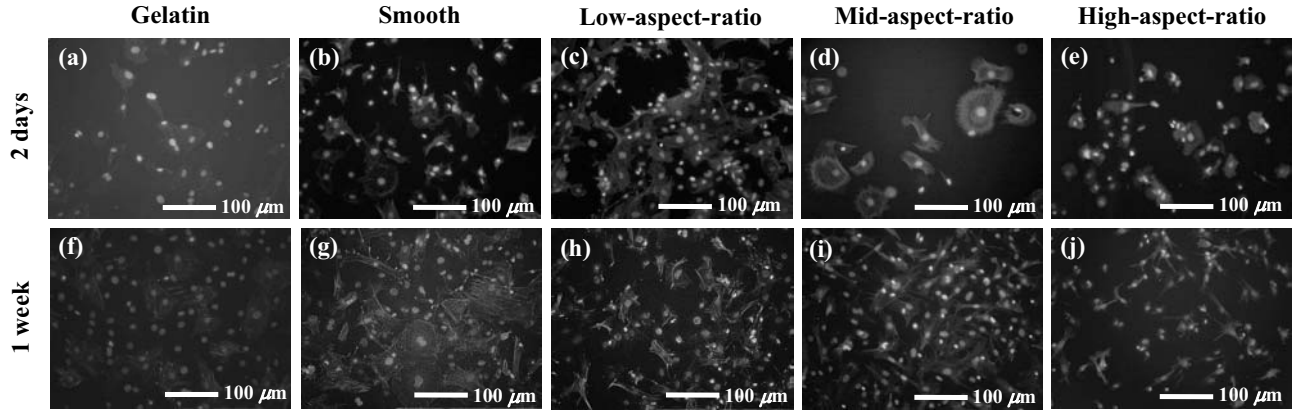

Figure 2: Fluorescence microscopy pictures of fibroblasts cultured for 2 days and 1 week on gelatin-coated, smooth, and NanoTurf surfaces. NanoTurf surfaces have three different aspect ratios: low, mid, and high, which correspond to Fig. 1(b), 1(c), and 1(d), respectively. Blue and red in colors represent nuclei and actins, respectively.

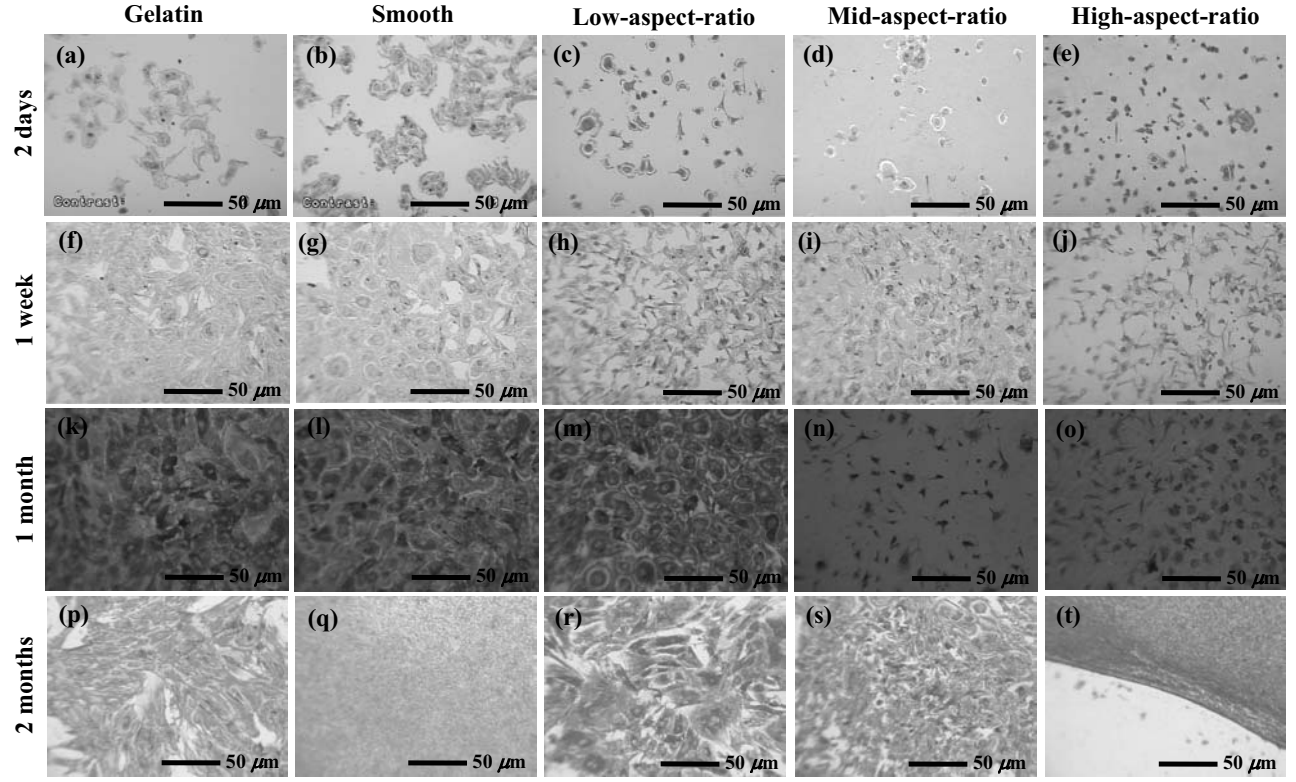

Figure 3: Bright field microscopy pictures of fibroblasts cultured for from 2 days to 2 months on gelatin-coated, smooth, and NanoTurf surfaces. NanoTurf surfaces have three different aspect ratios: low, mid, and high, which correspond to Fig. 1(b), 1(c), and 1(d), respectively.

streptomycin (100 U/ml, Invitrogen). The fibroblasts, i.e., non-cardiomyocytes, from the final digestion were plated for further cell culture analysis. The cells were kept at 37ºC and 5% CO_2. At confluence, the fibroblasts detached from the culture dishes by tyrosine/EDTA (Invitrogen) followed by centrifugation (1000 rpm, 5 minutes). The pellet was re-suspended in the culture medium. The neonatal rat fibroblasts at passage one were seeded at the density of 2×10^4 cm^{-2} on all three NanoTurf samples (1cm × 1cm) and two control surfaces (silicon and gelatin-coated silicon; both non-DRIE-modified). Before the cell seeding, every sample except the gelatin-coated surface was cleaned and made hydrophilic by Piranha solution (H_2SO_4:H_2O_2, 3:1 in volume). Cells were kept in culture for 2 days, 1, 4 and 8 weeks.

3. RESULTS AND DISCUSSIONS

Cell Morphology and Proliferation

The cultured cells were stained, or fixed and dried to be inspected by light microscopy and SEM. Figures 2 and 3 show the adhesion of fibroblasts on each sample at several culture periods. Although there were slight changes in cell populations on different surface conditions and culture periods, all surfaces supported good cell adhesion. Cells attached similarly well to gelatin-coated and non-coated silicon surfaces, suggesting that the bare silicon surface, strictly speaking native silicon oxide on the silicon surface, is at least as permissive to fibroblast attachment as gelatin. Cells on both of these smooth control surfaces exhibited flattened and rounded cell morphology as they spreaded. In contrast, all NanoTurf surfaces induced spindle-shape cell morphology. In particular, the cell shape became more slender and elongated with increasing aspect ratio of NanoTurf structures.

Fibroblasts were also cultured for a long period to see if they can proliferate enough to form a three dimensional sheet of cells over NanoTurf surfaces that could be peeled off for cell-sheet based tissue engineering [18]. Although the proliferation rate and the size of cells were slightly lower on the NanoTurf surfaces (especially on the mid- and the high-aspect-ratio structures) than the gelatin-coated and the smooth surfaces, a sheet of cells formed eventually on all the surfaces after more than one month. The boundary of the cell sheet formed over the high-aspect-ratio NanoTurf after two months is represented in Fig. 3(t) to show the edge of the cell sheet. The edge curled up during processing, while edges from smooth surfaces remained attached, suggesting that total adhesion force of the high-aspect-ratio NanoTurf to the cell sheet was not as large as that of the smooth surface. Although quantitative measurements of the adhesion force between the cell sheet and each sample were not performed, a simple and quick test of detachment of the cell sheet was done. The cell sheet grown on the high-aspect-ratio NanoTurf was easier to be peeled off than that on the smooth surface. These results suggest that nano-structured surfaces may facilitate cell sheet fabrication and removal for cell sheet engineering [18]. The controllability of the conventional cell sheet engineering by manual scrapping, or by thermoresponsive polymers to regulate surface wettability and the corresponding adhesion force, is very limited, depending on the polymer layer thickness. One additional advantage of nano-structured surfaces is that the adhesion force may be controlled by designing the nanostructure size and tip shape, without additional polymer coating and heat treatment, which may not be desirable in certain cases.

Cell Levitation and Extension

The adhesion force of the cell or cell sheet to the surface will be affected by the configuration of cell attachment over the surface. Due to the nanoscale pitch of the structures, the levitation of cells of a microscale size is expected over NanoTurf surfaces [8]. Figure 5 shows the levitation of lamellipodia and filopodia mostly observed over the mid- and the high-aspect-ratio NanoTurf surfaces, but much less over the low-aspect-ratio NanoTurf or the smooth surface, suggesting that the cell levitation is dependent on the aspect ratio of the NanoTurf. The easier detachment of the cell sheet over the high-aspect-ratio NanoTurf than the smooth surface can be explained by the very small fraction of the contact area of the cell to the solid surface due to the leviation by the high-aspect-ratio nanostructures. In addition to the levitation, more detail of how cells extend over the NanoTurf surfaces were also investigated. Figure 6 shows that filopodia prefer to advance along the sharp tips of structures when they extend. The sharp tips of nanostructures work as "stepping stones" in the filopodia movement, suggesting that filopodia can "sense" the nanoscale environment when they extend. Fundamental aspects of cell attachment and migration can be investigated using these NanoTurf surfaces.

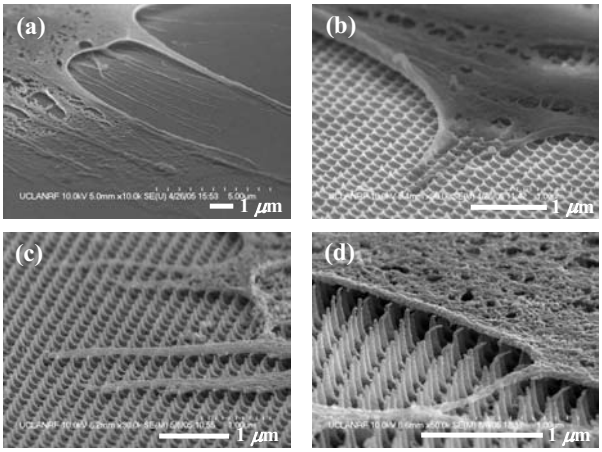

Figure 5: SEM pictures of levitation of lamellipodia and filopodia of fibroblasts cultured for 1 week. On (a) Smooth surface, (b) Low-aspect-ratio NanoTurf, (c) Middle-aspect-ratio NanoTurf, (d) High-aspect-ratio NanoTurf.

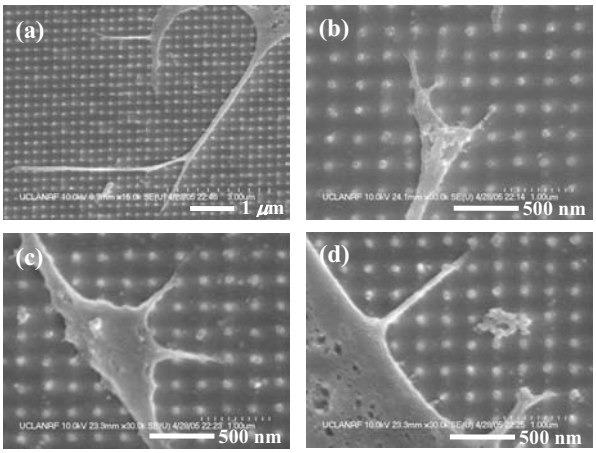

Figure 6: SEM pictures of extension of lamellipodia and filopodia of fibroblasts cultured on low-aspect-ratio NanoTurf for 1 week.

Cell Sheet and Matrix Formation

Cells typically synthesize their own extracellular matrix materials to modify their micro- and nano-environment. Figure 7 shows the cross sections of the cell sheets formed over a smooth and a high-aspect-ratio NanoTurf surfaces after culturing for 2 months. Accumulation of matrix meshwork was observed over a smooth surface, but little over the high-aspect-ratio NanoTurf. Although this difference needs yet to be explained with more exhaustive studies, it may be speculated that cells sensed the nano-posts as matrix structures so that they did not produce redundant matrix materials. Alternatively, the spindle shaped morphology on NanoTurf surfaces may be less optimized for extracellular matrix production, or the sharp-tip nanostructures may deactivate the production of the matrix materials by signaling the associated functions into the cells.

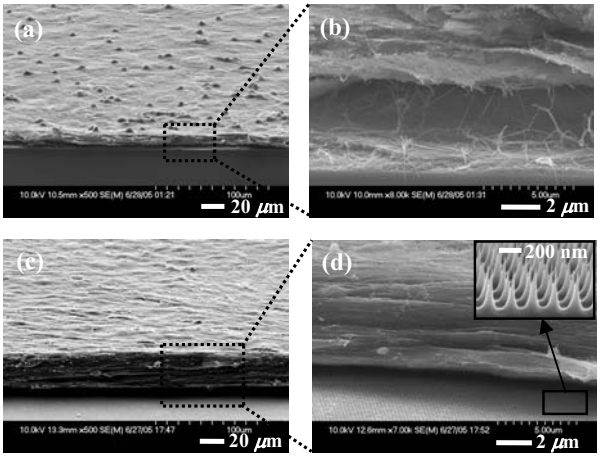

Figure 7: SEM pictures of a cross section of the sheet of fibroblast cells formed after culturing for 2 months. On (a)-(b) Smooth surface, (c)-(d) High-aspect-ratio NanoTurf.

4. CONCLUSIONS

Cells live in a nano- and micro-featured environment. Cells are sensitive to the environment that they may interact with objects as small as a few nanometers. Therefore, we need to know more about the reactions of cells to the nano-world and how to control them. One of the main practical ends of studies of cell behaviors on micro- or nano-structured surfaces is to be able to understand how cells attach and migrate in the body in three dimensional structures. We demonstrated that various nanostructure systems with good size and shape control can serve as a new means to elucidate the 3D cell-matrix interactions. A clear difference between a smooth surface and 3D NanoTurf surfaces on cell adhesions in various respects was also shown. Currently more details of cell adhesion mechanisms on the NanoTurf surfaces are being investigated using immunohistochemistry specific for focal adhesion kinase and integrin $\alpha_5\beta_1$. These results can be further utilized to design specific functions in biomedical devices dealing with cells. Several tissue engineering applications of the NanoTurf surfaces can also be envisioned [19].

5. ACKNOWLEDGEMENTS

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