Comparison of cell interactions with laser machined micron- and nanoscale features in polymer

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Abstract

Control of cell responses to artificial surfaces is a research goal for much of the biomaterials community. The role that the micron scale topography of a surface can play in controlling cell responses has been well documented and recent advances in nanofabrication techniques have lead to an interest in cells' responses to submicron-scale surface features. The study described here compares the relative influences that nanoscale and micron-scale features exert on cells by examining cytoskeletal organisation. Micron-scale structures were generated on the polyamide Kapton® using a 193 nm ArF Excimer laser, at 400 mJ/cm^2 fluence. Nanoscale features were generated on Kapton using the excimer laser with a phase mask. Osteoblasts were seeded onto surfaces for 24 h, then the cell membranes were detergent-extracted, and the cells were applied with a primary antibody to actin and a colloidal gold-conjugated secondary antibody. Samples to be examined using the confocal were mounted in glycerol, those for electron microscopy were carbon-coated. The organisation of actin was examined on micron- and nano-scale structures by scoring sections for order of branching and angles of branching to relate changes in the cytoskeleton relative to the control. Although there was a strong influence of micron-scale structures, the cytoskeleton of cells on the nanoscale structures were similar to the controls.
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Introduction

The field of biomaterials involves examining the interactions of cells and tissues with artificial substrates used as medical devices or implants. Control of the cell responses to materials is a research goal for much of the biomaterials community. While research has highlighted the role that micron-scale surface topography plays in influencing cell responses (Anselme et al., 2000a; Barbucci et al., 2003; Curtis, 1997, 2001; Jiang et al., 2002; Lincks et al., 1998), recent advances in nanofabrication and imaging systems have lead to an increasing number of studies examining the influence of nano-scale topography on cell responses (Chin et al., 2004; Kriparamanan et al., 2006; Liliensiek et al., 2006; Sniadecki et al., 2004).

In the light of these reports, a number of researchers have attempted to optimise existing materials by machining them or generating structures on their surfaces. Recent reports have shown that the laser shows promise for altering surface chemistry (Ball et al., 2004) for biomaterial applications. In this study we utilised the laser to generate micron- and nanometre-scale surface features. In examining the reactions of cells to surface topography, much attention is given to orientation of the cytoskeleton and cell adhesion to the contours of the substrates (Chou et al., 1995; Anselme et al., 2000b). This has been largely performed by using antibodies to cytoskeletal proteins such as actin, labelled with fluorophores. The cells are then examined using fluorescent microscopy. To be able to fully consider the effect of the surface on the positioning of cytoskeletal or adhesion proteins, it is useful to be able to view the surface and cellular components in the same visual field. While this is possible with micron-scale features, it is problematic with nano-scale features. The size of nanostructured features stretches the resolving power of conventional

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light and fluorescence microscopy to their limits. One possible solution may be using scanning electron microscopy. Scanning electron microscopy (SEM) can offer the resolution required to examine nanostructured materials, as well as allowing visualisation of the substrate and cell at the same time. Conventional scanning electron microscopy cannot be used to detect the same fluorophores as used in fluorescent microscopy, however. Instead, gold can be used as a substitute for a fluorescent dye. Recent advances in the use of immunoglobulin conjugated with colloidal gold has shown considerable promise in allowing the possibility of labelling proteins with sufficient contrast to be viewed with electron microscopy (Hermann et al., 1996; Richards et al., 2001). In this study, we used a primary antibody to β-actin, conjugated to an immunogold label. The techniques used are derived from those previously developed for labelling cells for examination using transmission electron microscopy (Svitkina and Borisy, 1998). The cell membrane was detergent-extracted, followed by the application of an antibody similar to the protocol used in conventional immunofluorescence labelling techniques. The gold particles were later enlarged using gold enhancement. Silver enhancement of gold particles is a commonly technique for enhancing, the size, and hence the ease of detection, of gold nanoparticles. Gold enhancement uses a gold solution which binds covalently to immunoglobulin-bound gold. Gold gives a stronger backscatter signal than silver and will not react with heavy metal salts such as osmium tetroxide (Owen et al., 2001).

Methods

Generation of structures

The polyamide polymer Kapton® (Goodfellow, Ireland) was modified using a 193 nm ArF Excimer laser, at 400 mJ/cm^2 fluence, generating micron-scale surface features. Features generated were 15 μm wide, 20 μm deep and had a 20 μm period. Nano-scale features were generated on Kapton using the excimer laser with a phase mask in a −1/+1 configuration. The phase mask consisted of a grating etched in fused silica and was separated from the sample using a 50-μm Kapton spacer. As the beam interacts, it created an interference pattern with a period one half of that of the phase mask. Using three laser pulses at a fluence of 175 mJ/cm^2, features were created with a period of ∼630 nm and a depth of ∼320 nm.

Seeding and staining of cells

SaOs-2 osteoblast-like cells (ECACC, Porton Down, Wiltshire, UK) were cultured in glutamine-free McCoys 5A media (Sigma-Aldrich, Tallaght, Dublin, Ireland) supplemented with 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin solution (all GIBCO BRL, Dublin, Ireland). The cells were seeded onto surfaces at a density of 200 cells/mm^2 in sterile 24-well plates (Sarstedt). After culturing for 24 h, the cells were removed and processed for scanning electron microscopy (SEM).

Preparation of cells

Cells were processed for scanning electron microscopy using a modification of a method used for immunogold labelling of cytoskeletal components for TEM (Svitkina and Borisy, 1998). After an initial rinse using 0.1 M phosphate-buffered saline (PBS), pH 7.4, the cell membranes were detergent extracted immediately, by incubating cells for 5 min at 20 °C with an extraction solution (1% Triton X-100 (Sigma-Aldrich, Dublin, Ireland), 4% polyethylene glycol, M_w=40,000 (Serva, Heidelberg, Germany) in cytoskeleton buffer (50 mM

imidazole, pH 6.8, 50 mM KCl, 0.5 mM MgCl$_2$, 0.1 mM EDTA, 1 mM EGTA) (all Sigma-Aldrich, Dublin, Ireland). The extraction buffer was supplemented with 10 μg/ml taxol to stabilise cell microtubules. Samples were then rinsed for 5 min in cytoskeleton buffer, and stained using rabbit anti-sera to actin (Sigma-Aldrich). These were then rinsed three times for 5 min each in cytoskeleton buffer, and fixed for 20 min with 2.5% glutaraldehyde (Agar Scientific, UK), in 0.1 M sodium cacodylate buffer, pH 7.3 (Sigma-Aldrich). Following this, the cells were quenched twice using 50 mM glycine (Sigma-Aldrich) for 10 min each, rinsed firstly with 0.1 M phosphate-buffered saline, pH 7.4, three times and then with 0.5 M NaCl, 0.05% Tween, 0.1% bovine serum albumin in 20 mM Tris–HCl, pH 8 (all Sigma-Aldrich). The secondary antibody used was an anti-rabbit IgG, conjugated both with Alexa fluor 488 and a colloidal gold label (Nanogold®, Molecular Probes, Invitrogen, UK), and was diluted in the Tris/NaCl/Tween buffer with 1% BSA at 1:10. The cells were incubated with the secondary antibody overnight at 4 °C. After incubation, the secondary antibody was removed, the cells rinsed with cytoskeleton buffer, then fixed with 2.5% glutaraldeyde in 0.1 M sodium cacodylate for 20 min at room temperature (RT). After fixation, the cells were rinsed three times with PBS supplemented with 50 mM glycine. Samples were rinsed with distilled water three times and treated with GoldEnhance (Nanoprobes Inc, Universal Biologicals, Cambridge, UK) for 8 min to increase the size of gold nanoparticles. After treatment, the GoldEnhance solution was removed and the cells treated with 0.1% tannic acid for 20 min at RT. Cells were then rinsed with distilled water twice, with a further incubation with distilled water for 5 min at RT. Samples were dehydrated using a progressive series of alcohols and finally with hexamethyldisilazane (HMDS) (Sigma-Aldrich). Samples were carbon coated prior to viewing using a Hitachi S-4700 FE-SEM. Samples were viewed at working distances of 4–6 mm, using 3–5 kV in secondary electron mode with a −150 mV bias to image the gold label.

For comparison samples were stained with TRITC-conjugated phalloidin. Fluorescently labeled samples were viewed using a Zeiss LSM 310 confocal laser scanning microscope.

Scoring of cytoskeleton

The organisation of actin was examined on flat, micron- and nano-scale structures by scoring sections for order of branching and angles of branching to relate changes in the cytoskeleton to the control. Three sample areas from separate images were selected at random and viewed at 500× magnification. Images of cells visible within that field were taken at 3500× magnification. The subsequent SEM images were examined using the ImageJ image processing package (National Institute of Health, US). The starting point for measurements was taken as the point at which actin fibres connected with the cell membrane. The actin fibre at this point was considered to be the primary (first-order) branch. Any fibres that split from the first-order branch were scored as a second-order branch. The angle from the first-order branch created by the second-order branch was measured using the angle measuring tool on ImageJ. The second-order branch was followed, and any fibres that split from this branch were scored as third-order branches. The angle this fibre formed was also measured. This was continued for the whole field of view, with subsequent deviations scored as fourth-, fifth- or higher-order branches as appropriate, and the angles made by the branch with the parent being measured.

Statistics

Data was processed using a one-way analysis of variance using Tukey's post-test, n=5, with a statistically significant difference set at p<0.001.

Results

In this study, we compared the cytoskeleton of SaOs-2 cells on both flat and grooved Kapton surfaces by use of scanning electron microscopy and immunogold labelling. Use of a bias voltage allows much clearer visualisation of the stained portions of the cytoskeleton. On flat (i.e. non-laser-treated) Kapton, SaOs-2 cells showed typical spread morphology. On viewing