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Biomimetic microtopography to enhance osteogenesis *in vitro*

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ABSTRACT

Biomimicry is being used in the next generation of biomaterials. Tuning material surface features such as chemistry, stiffness and topography allow the control of cell adhesion, proliferation, growth and differentiation. Here, microtopographical features with nanoscale depths, similar in scale to osteoclast resorption pits, were used to promote *in vitro* bone formation in basal medium. Primary human osteoblasts were used to represent an orthopaedically relevant cell type and analysis of adhesions, cytoskeleton, osteospecific proteins (phospho-Runx2 and osteopontin) and mineralisation (alizarin red) was performed. The results further demonstrate the potential for biomimicry in material design and show that the osteoblast response can be tuned from changes in feature size.

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1. Introduction

It has been known for a century that cells will respond to the shape of their environment [1]. Over the last few decades, because of the use of miniaturization techniques borrowed from the microelectronics industry, the effects of surface microtopography on a range of materials have become further elucidated [2]. Such effects include alterations in cell adhesion, migration, cytoskeletal organisation, genome regulation and even differentiation [3–9]. More recently it has been revealed that nanoscale topographies can alter cell response, similarly driving large changes in adhesion, genomic regulation and differentiation [10–16].

This is interesting as microscale features are on a similar scale to the cells themselves, so it is easy to see how irresistible cues are presented to the cells that they must follow due to physical containment of microscale groove contact guidance. Nanoscale features, however, are orders of magnitude smaller than most mammalian cells, e.g. osteoblasts. The features are, in fact, at the same scale as filopodia, which are actin-driven membrane projections (50–100 nm diameter tips) that cells use to probe the surface [17–20], and are even on a similar scale to individual cell receptors, e.g. integrins. For cells to adhere, integrins must first bind to their peptide ligands (e.g. RGD) and then gather together into larger focal adhesions. These adhesions act as anchors for the contractile actin, seen in cells as stress fibres. Such is the nanoscale sensitivity of the integrins that if their spacing is beyond 70 nm from each other they cannot gather together and focal adhesion does not take place. Hence actin polymerisation is prevented and the intracellular tension required for differentiation cannot be established [21–24]. It is noted that a material's surface needs to recruit sufficient extracellular adhesion proteins (such as fibronectin) to allow integrin gathering or the cells can detach during the adhesion process.

There has been a move towards biomimicry in eliciting influence over osteogenesis using materials. Such approaches can be chemical, using adhesive proteins to induce cell spreading [25,26], or physical, using tissue-matching stiffness, causing cells to either retain cytoskeletal tension (producing osteogenesis from mesenchymal stem cells) on stiff substrates or dissipate their tension to the substrate (producing myogenesis or even adipogenesis from mesenchymal stem cells) on soft substrates [27]. A nanotopographical approach has also been described where, rather than the traditional approaches of using exact order (as produced by electron beam lithography, EBL) or randomness (as produced by e.g. anodization or blasting), controlled disorder was used (EBL patterning) and high levels of mesenchymal stem cell osteoinduction were observed [15].

There have been recent reports that a mixed micro/nanoscale approach to mimicking features with similar dimensions to

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osteoclast bared resorption pits may be osteogenic [28]. Here, we use photolithography to fabricate features that are then embossed into polycaprolactone (PCL). (Note that PCL is not the intended material for clinical use; rather, we are assessing the bioactivity of the topographical features for use in a variety of potential materials.) The pit features have diameters of 20, 30 and 40 μm and fixed depths of 300 nm. Primary human osteoblasts have been used to test the materials using histological analysis for cytoskeleton, adhesion, protein markers and calcium deposition.

The hypothesis for the study is that the use of biomimicry, i.e. replicating features of a size osteoblasts would perceive as representing osteoclast activity, would stimulate bone production. Our intention is to develop bioactive patterns that can then be translated to implant materials where osteogenesis would be of benefit.

2. Materials and methods

2.1. Materials

Silicon wafers (Compart Technologies, UK) were cleaned under acetone in an ultrasonic bath for 5 min. They were then rinsed thoroughly in reverse osmosis water (ROH_2O) [29] and blow dried with an air gun. Next, they were spun with primer (Shipley AZ Coupler, Shipley, UK) for 30 s at 4000 rpm, then spun with S1818 photoresist (Shipley, UK) for 30 s at 4000 rpm and baked for 30 min at 90 °C. The resulting layer was measured to be 1.8 μm thick. The photoresist layer was exposed to ultraviolet light through a chrome mask on a Karl Suss MA6 mask aligner for 3.8 s, then the resist layer was developed for 75 s in 50:50 Microposit developer (Shipley, UK): ROH_2O.

The developed circle patterns were then used as a mask for reactive ion etching. The silicon substrate was etched in the silicon tetrachloride gas plasma of a Plasmalab System 100 machine (gas flow = 18 sccm, pressure = 9 mT, rf power = 250 W, DC bias = −300 V). Each wafer was etched individually at 18 min at a nominal etch rate of 18 nm min^{-1}. All three wafers were stripped of resist in an acetone ultrasound bath for 5 min, followed by a 5 min soak in concentrated sulphuric acid/hydrogen peroxide mixture before being rinsed thoroughly in ROH_2O and dried in an air gun.

Nickel dies were made directly from the patterned resist samples. A thin (50 nm) layer of Ni–V was sputter coated on the samples. This layer acted as an electrode in the subsequent electroplating process. The dies were plated to a thickness of ca. 300 μm. Once returned from the plater, the nickel shims were cleaned by first stripping the protective polyurethane coating using chloroform in an ultrasound bath for 10–15 min. Second, silicon residue was stripped by being wet etched in 25% potassium hydroxide at 80 °C for 1 h. Shims were rinsed thoroughly in ROH_2O and then air-gun dried. The shims were finally trimmed to approximately 30 × 30 mm sizes using a metal guillotine [30].

Imprinting of the nickel shims into polymethylmethacrylate (characterisation) or polycaprolactone (M_n = 60,000 used for bioanalysis) was used to allow rapid replication for analysis. For polymethylmethacrylate, embossing was achieved using an Obducat (Obducat, Sweden) nanoimprinter (temperature = 180 °C, pressure = 15 bar, time = 300 s). The imprints were trimmed and then depth measurements (Dektak, Bruker, UK) were made of a random sample from each etch depth category. Random samples were taken from each depth category for inspection by scanning electron microscopy (SEM). For polycaprolactone, hot embossing at 70 °C was used to imprint the substrates. Controls were fabricated by injection moulding/embossing against planar shims. Materials were washed/sterilized in 70% EtOH and then in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) saline prior to cell seeding. The materials were fabricated as 1 cm diameter discs (approx. 0.5 mm thick) to fit snugly into 24-well plates.

2.2. Cell culture

All experiments were performed with adult human osteoblastic cells after 1–4 passages *in vitro* (purchased from PromoCell™, Heidelberg, Germany). Cells, seeded at a density of 1×10^4 cells/ml (hence 1×10^4 cells/0.79 cm^2) were cultured in basal medium (alpha-minimum essential medium (αMEM) with 10% foetal calf serum (FCS), InVitrogen, UK), which was changed twice each week. Each test was performed with three material replicates and each test was performed with cells from the same patient.

2.3. Immunofluorescence

After 3 (focal adhesions and cytoskeleton) or 21 (osteopontin) days of culture, the cells on the test materials were fixed in 4% formaldehyde/phosphate-buffered saline (PBS) with 1% sucrose at 37°C for 15 min. When fixed, the samples were washed with PBS and a permeabilizing buffer (10.3 g of sucrose, 0.292 g of NaCl, 0.06 g of $MgCl_2$, 0.476 g of HEPES buffer, 0.5 ml of Triton X, in 100 ml of water, pH 7.2) added at 4 °C for 5 min. The samples were then incubated at 37 °C for 5 min in 1% bovine serum albumin (BSA)/PBS, followed by the addition of one of anti-vinculin, anti-β tubulin primary or anti-osteopontin antibody (1:100 in 1% BSA/PBS, h-vin1 (vinculin, Sigma, UK), tub 2.1 (tubulin, Sigma, UK) or AKm2A1 (osteopontin, Autogen Bioclear, UK) monoclonal anti-human antibody raised in mouse (IgG_1)) for 1 h (37 °C). Simultaneously, rhodamine-conjugated phalloidin was added for the duration of this incubation (1:100 in 1% BSA/PBS, Invitrogen, UK). The samples were next washed in 0.5% Tween 20/PBS (3× for 5 min). A secondary, biotin-conjugated antibody (1:50 in 1% BSA/PBS, monoclonal horse anti-mouse (IgG), Vector Laboratories, Peterborough, UK) was added for 1 h (37 °C) followed by washing. A third, fluorescein isothiocyanate-conjugated streptavidin, layer was added (1:50 in 1% BSA/PBS, Vector Laboratories, Peterborough, UK) at 4 °C for 30 min, before the samples were given a final wash. They were then viewed with a fluorescence microscope (Zeiss Axiovert 200M, 40× magnification, NA 0.5).

2.4. Alizarin red histology

Alizarin red stain (pH 4) of 2% w/v was prepared by mixing 2 g of alizarin red S (Sigma) with 100 ml of water and dilute ammonium hydroxide was added to adjust the pH. After 28 days of culture, the cells were fixed in 4% formaldehyde for 15 min at 37 °C. Then they were stained with 2% alizarin red for 5 min before washing with tap water (3× for 1 min). Samples were then counterstained for 10 s in 0.5% Coomassie blue in a methanol/acetic acid aqueous solution, and washed (3× for 1 min) again with water. Samples were viewed by bright-field optical microscopy (10× magnification, NA 0.3). Pictures were taken with a greyscale digital camera (Scion Corporation Model CFW-1310M).

2.5. Statistics

The stained areas of the alizarin red-stained samples (as described above) were manually counted in 0.5 cm^2 areas. Statistics were calculated by analysis of variance (ANOVA) using Sigma Stat (Systat Software, UK).

2.6. Scanning electron microscopy

After 28 days of culture, cells were fixed in 4% paraformaldehyde and air dried. After drying, samples were sputter coated

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