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Fabrication of pillar-like titania nanostructures on titanium and their interactions with human skeletal stem cells

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Abstract

Surface nanotopography is known to influence the interaction of human skeletal (mesenchymal) stem cells (hMSC) with a material surface. While most surface nanopatterning has been performed on polymer-based surfaces there is a need for techniques to produce well-defined topography features with tuneable sizes on relevant load-bearing implant materials such as titanium (Ti). In this study titania nanopillar structures with heights of either 15, 55 or 100 nm were produced on Ti surfaces using anodization through a porous alumina mask. The influence of the surface structure heights on hMSC adhesion, spreading, cytoskeletal formation and differentiation was examined. The 15 nm high topography features resulted in the greatest cell response with bone matrix nodule forming on the Ti surface after 21 days.

Keywords: Titanium; Nanotopography; Mesenchymal stem cell; In vitro test

1. Introduction

Exquisitely small changes in nanotopography have been shown to influence mesenchymal stem cell differentiation to form bone with similar efficiency to chemical treatments, with implications therein for applications in orthopaedics and dentistry [1]. However, to date, the translation from in vitro promise to practical delivery of novel implant materials has been hampered by an inability to pattern in relevant load-bearing materials. Titanium and its alloys remain the most commonly used implant materials in orthopaedics and dentistry. However, in vivo these materials may form a fibrous capsule at the Ti surface, rather than the desired direct bone bonding, leading to micromotion and, ultimately, failure [2].

Surface treatments, such as topographical modification, have the potential to produce bioactivity without affecting the mechanical characteristics of the material. However, traditional precision nanofabrication, such as electron beam lithography [1,3], polymer demixing [4,5] and colloidal lithography [6], have been used on polymer-based materials that have a far lower Young's modulus than Ti. Most techniques used to produce nanotopography on Ti, such as acid etching [7], deposition of TiO_x clusters [8], layer-by-layer assembly [9] and anodization [10], create various degrees of surface nanoroughness but lack precise control and tuneability of the topographic features. It remains difficult to deduce the precise effects of surface topography when roughness is composed of randomly arranged surface features and, also, the morphology of surface topography may influence cellular reactions to a greater degree than roughness values [11].

In this work we aimed to evaluate the response of human mesenchymal stem cells (hMSCs), or skeletal stem

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cells, to Ti surfaces with reproducible oxide topographic features of various heights from 15 to 100 nm. hMSCs have been shown to be sensitive to topographical packing (i.e., random through to ordered arrangement) differences below 20 nm [1]. The smallest features cells have been shown to interact with have been 10 nm in height [12]. Furthermore, the fibroblast cell response has been shown to be tunable when interacting with features between 20 nm (high cell activity) and 100 nm (low cell activity) in height with intermediate states in between [13]. Whilst we are primarily interested in bone formation, we note that hMSCs can give rise to cells of the stromal lineage, namely adipogenic (fat), chondrogenic (cartilage), osteoblastic (bone), myoblastic (muscle) and fibroblastic (connective tissue) lineages, and can generate intermediate progenitors and thus lineage modulation remains a further objective [14].

To fabricate surfaces with well-defined nanosized topographic features we have used an anodization technique where a porous anodic alumina mask is used as a template on a Ti surface to pattern the Ti with self-arranged dot- and pillar-like nanostructures. In essence, the technique utilizes the formation of porous anodic alumina, whereby large arrays of well-ordered, nanosized alumina pores with tunable dimensions are produced [15–18]. When aluminium is deposited and anodized on top of a Ti substrate, the Ti is subsequently anodized through the alumina pore bottoms with the nanosized pattern of the pores inherited on the substrate. Although this technique has previously been used to pattern thin layers (5–300 nm) of metal which had first been sputter deposited on glass or silicon wafer substrates [19–22], it is the first time the technique has been shown to work directly on a bulk Ti surface, as would be suitable for dental and orthopaedic implants.

2. Materials and methods

2.1. Surface fabrication

Ti samples with a diameter of 11 mm were cut from a 0.3 mm thick sheet of Ti (Goodfellow, 99.6 + % purity) and mechanically polished to a mirror surface. An Al layer (~1 μm) was deposited on the polished Ti surfaces using a Balzer 510 coater system. The Al source had a purity of 99.99%. To anodize the samples a copper wire was fastened to the uncoated side of the Ti disks. The wire and the uncoated Ti were then sealed with a rapid curing epoxy to ensure that only the area coated with Al was exposed to the electrolyte. An Agilent N5752A DC power supply was used as the voltage source and a Keithley 2000 multimeter was used to monitor the current over the electrochemical cell during the anodizations. Both the power supply and the multimeter were controlled via a computer. The electrochemical cell consisted of a glass beaker placed in a temperature controlled water bath and the electrolyte was stirred with a magnetic stirrer throughout the anodizations. A platinum strip was used as the cathode. All the anodizations were performed in 0.3 M oxalic acid at 17 °C. They were performed with constant voltage until the Al had been completely consumed, indicated by a rapid current decrease. The process was then either interrupted or the voltage was increased in order to increase the height of the Ti oxide. The samples were anodized at 15 V/15 V, 30 V/40 V or 45 V/70 V (first voltage used during anodization of the Al layer and second voltage used to further anodize Ti after complete Al anodization). After anodization the samples were rinsed in distilled water and the epoxy was carefully removed from the back of the samples. Finally, to reveal the titania structures the alumina mask was chemically removed in a solution containing 6 wt.% H_3PO_4 + 1.8 wt.% CrO_3 at 60 °C for 15 min. Field-emission scanning electron microscopy (JEOL JSM 6330F) and atomic force microscopy (Veeco Multimode with Quadrex Nanoscope 3D) were used to characterize the morphology of the samples.

2.2. Human skeletal (mesenchymal) stem cells

Human osteoprogenitors (all adherent cells of the bone marrow) were extracted from bone marrow samples obtained from haematologically normal patients undergoing routine hip replacement surgery as described previously [23]. Osteoprogenitor cells, including hMSCs, were selected using stro-1 antibody and magnetic cell sorting (MACS) [24,25], as previously described [23]. hMSCs were maintained in a basal media (10% foetal calf serum/α-modified Eagle's medium, Life Technologies, UK). Cells were seeded onto the materials at 1×10^4 cells per sample and the media changed twice weekly. All cells in the study were used at passage 1 or 2.

2.3. Immunofluorescence

After 4 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 sucrose, 0.292 g NaCl, 0.06 g $MgCl_2$, 0.476 g HEPES buffer, 0.5 ml Triton X, in 100 ml water, pH 7.2) was 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 either an anti-vinculin, anti-vimentin or anti-β tubulin primary antibody (1:100 in 1% BSA/PBS, h-vin1 (vinculin), V9 (vimentin) or tub 2.1 (tubulin) monoclonal anti-human raised in mouse (IgG1), Sigma, Poole, UK) for 1 h (37 °C). Simultaneously, rhodamine conjugated phalloidin was added for the duration of this incubation (1:100 in 1% BSA/PBS, Molecular Probes, Oregon, USA). The samples were next washed in 0.5% Tween 20/PBS (3 × 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 FITC conjugated streptavidin third layer was added (1:50 in 1% BSA/PBS, Vector Laboratories, Peterborough, UK) at 4 °C for 30 min, and given a final wash.

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