Journal of the mechanical behavior of biomedical materials 68 (2017) 26–31

Contents lists available at ScienceDirect

Journal of the Mechanical Behavior of Biomedical Materials

journal homepage: www.elsevier.com/locate/jmbbm

Research Paper

Modulation of osteoblast behavior on nanopatterned yttria-stabilized zirconia surfaces

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ARTICLE INFO

Keywords:
Nanostructure
Osteoblast
Zirconia
Cell behaviour
Self-assembly

ABSTRACT

This study utilizes the technique of self-assembly to fabricate arrays of nanoislands on (001)-oriented yttria-stabilized zirconia single crystal substrates with miscut of 10° toward < 110 > direction. These self-assembled nanostructures were annealed at 1100 °C for 5 h upon doping with 10 mol% gadolinium-doped ceria (GDC) by powder-suspension based method. X-Ray diffraction result showed that the miscut substrate after doping GDC was in the cubic phase. Energy dispersive X-ray (EDX) illustrates that the nanopatterned material contains all the elements from the GDC source and yttria-stabilized zirconia (YSZ) substrate. It also demonstrates a higher surface roughness and a more hydrophilic surface. The nanostructured materials were subsequently used for an *in vitro* study using a human fetal osteoblastic cell line (hFOB). An improved spreading, enhanced cell proliferation and up-regulated alkaline phosphatase activity (ALP) were observed on the nanopatterned substrates compared to the control substrates. Calcium deposits, which were stained positively by Alizarin Red S, appeared to be more abundant on the nanopatterned surfaces on day 7. The overall findings suggest that post fabrication treatment with surface modification such as creating a nanostructure (e.g. nanopatterns) can improve biocompatibility.

1. Introduction

New discoveries and advances in nanotechnology have allowed the fabrication of nanostructured biomaterials for a broad range of applications, including drug delivery (Zamani et al., 2013), biosensors (Q.Q. Sun et al., 2015; Tarlani et al., 2015), and tissue engineering or regenerative medicine (Kulkarni et al., 2015). Top-down and bottom-up approaches such as lithography (Richardson et al., 2011; Zhu et al., 2011), electrospinning (Jalaja et al., 2014) and self-assembly (Ansari et al., 2013; Rolandi and Rolandi, 2014) are common techniques to fabricate various structures with nano-dimensions. Cell–substrate interaction is a crucial factor in determining biocompatibility when designing medical implants. Surface features at a nanoscale provide direct mechanical interactions with cell receptors and components, in turn regulating the cells in order to fulfill their intended purposes. In particular, nano-features in tissue engineering applications are perceived by cells as microenvironment-like substances or cell microenvironments (An et al., 2013). The idea of using nanoscale surface features for biomaterials research has provided various feasible and versatile alternatives to studies of biological interactions between biomaterials at a cellular level and *in vivo* response.

Surface engineering of materials at a nanoscale allows topographical properties alone to induce different responses even for the same cell phenotype (Stevens and George, 2005). Surface modification at a nanoscale modulates the cell behavior at different levels, ranging from cell adhesion, orientation, and motility to alterations in intracellular signaling pathways (Stevens and George, 2005). Recent studies have established a variety of cellular responses to varied surface nanotopographies, including nanogrooves (Davidson et al., 2015), nanopits (Ni et al., 2014), nanoridges (X. Sun et al., 2015), and nanofibres (Choi et al., 2014). ECM consists of rich components with micro- or nanoscales such as protein fibres, collagens and elastins, which are able to provide cells with an abundance of signals (Dvir et al., 2011). Surface nanotopography can resemble the natural extracellular matrix (ECM), where cells reside in and interact with the ECM (Lord et al., 2010).

The contributions of surface microtopography to cellular behaviour have been well established and acknowledged in the past (Duncan et al., 2002; Keller et al., 2003). For example, surface microtopography dictates the way molecules are adsorbed to a surface, and the attachment and the alignment of cells on a surface (Boyan et al., 1996). Osteoblast-like cells tend to attach to rougher surfaces, which is

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http://dx.doi.org/10.1016/j.jmbbm.2017.01.028
Received 20 November 2016; Received in revised form 18 January 2017; Accepted 20 January 2017
Available online 21 January 2017
1751-6161/

the opposite of the behavior of epithelial cells and fibroblasts (Boyan et al., 1996). Besides cell attachment, proliferation and spreading, it has been demonstrated that nanotopography exerts intense effects on a variety of cell types in terms of focal adhesion complexes, gene expression alteration, and cell differentiation (Martínez et al., 2009). The additional advantages of surface nanotopography over microtopograhy have been highlighted in terms of affecting cell differentiation, in which nanotopographic cues induced significantly higher cell differentiation in comparison to microtopographical surfaces (Turner and Dalby, 2014). Additionally, surface roughness and wettability have also been studied for their influence on cell growth and behavior. Surface roughness was reported to affect the production of local growth factors, cell differentiation, and proliferation (Kieswetter et al., 1996; Martin et al., 1995). Wettability has also shown strong biological interaction characteristics, as it affects protein adsorption and subsequently cell adhesion (Rosales-Leal et al., 2010).

Zirconia ceramics offer several advantages as biomaterials, such as biocompatibility, and interesting mechanical properties (Piconi and Maccauro, 1999). Biomedical grade zirconia was introduced at first to overcome the problem of alumina bioceramics in terms of brittleness (Chevalier, 2006). Not only did zirconia as a biomaterial find application in hip joint replacement, it has also been introduced in the prosthetic dentistry as the base for restorative dental materials (Denry and Kelly, 2008). Nevertheless, clinical use of zirconia biomaterial is restricted due to the difficulty in fabricating modified surfaces (Depprich et al., 2008). Further, smooth surfaces are not beneficial for the process of osseointegration (Depprich et al., 2008). Although the utilization of single crystal YSZ for implant applications is not as common as polycrystalline YSZ, the employment of these nanostructures can be explored for various applications. For example, these nanostructures can be transferred to other polymers by nanoimprinting (Zimmerman et al., 2009). Large-scale arrays of nanoislands could also be reproduced on other larger substrates by applying the single crystal YSZ and GDC system. This study has utilized the fabricated arrays of self-assembled nanostructures which is a simple and low-cost method to investigate the interaction between osteoblasts and self-assembled nanoislands. The results have demonstrated a positive response of osteoblasts to the nanotopographical cues imposed, in terms of cell proliferation, adhesion, differentiation and mineralization.

2. Material and methods

2.1. Thermal annealing of YSZ with GDC suspension

Single crystal 8 mol% YSZ-(001) substrates with a 10° miscut towards [110] were purchased from MTI Corporation (USA). The substrates were 5.0 mm×5.0 mm×0.5 mm in dimensions and chemically polished as delivered. 0.1 g/L of 10 mol% GDC powder (Nextech Materials, USA) suspension was prepared in deionized water and ultrasonicated (Sonics & Materials, USA) for 5 min. The suspension was then applied to the substrates using a plastic pipette before annealing at 1100 °C for 5 h at a ramp rate of 10 °C min^{-1} in a box furnace (Nabertherm, Germany) (Ansari et al., 2013).

2.2. Characterization of YSZ substrate with nanoislands

Nanostructures were observed using field emission scanning electron microscopy (FESEM) (JEOL, Japan). Substrates were gold coated prior to FESEM imaging. Energy-dispersive X-ray spectroscopy (EDX) (Phenom) and X-ray diffractometry (XRD) (Empyrean, PANalytical, Netherlands) were used to determine the composition of the nanostructures using spot analysis and the phase purity of the nanostructured material, respectively. XRD operated at 45 kV and 40 mA with Cu-Kα as the radiation source. Step scan and scan speed were 0.02° and 0.5° min^{-1}, respectively with a 2Θ scanning range of 20°–50°. Surface roughness was assessed by atomic force microscopy (Ambios Technology, USA). Wettability, which is indicated by water contact angle, was analyzed by using a contact angle goniometer (Kruss, Germany). A wettable surface is also known to be a hydrophilic surface. A lower water contact angle indicates a more hydrophilic surface as water spreads out more on a hydrophilic surface.

2.3. Culture of human fetal osteoblast (hFOB 1.19, ATCC No.: CRL-11372)

Human fetal osteoblasts (hFOB 1.19, ATCC No: CRL-11372) were cultured at 34 °C under 5% CO_2 atmosphere according to the handling procedures provided by the manufacturer. The cells were grown in complete growth medium consisting of a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Biosera), 1% antibiotic-antimycotic (Gibco) and 0.3 g/L G418 (Gibco). Culture medium was changed every 3–4 days. The subculturing procedure was done by using tryPLE select (Gibco) and p4 osteoblasts were used for the study. Sterilization of samples for *in vitro* study was done by washing both the miscut substrates without nanoislands (control samples) and the miscut substrates with nanoislands (nanopatterned samples) with 70% ethanol, followed by rinsing with phosphate buffered saline (PBS; Sigma-Aldrich), prior to ultraviolet disinfection on both sides for 1 h. Cells were seeded at 10,000 cells/cm^2 on both control and nanopatterned samples for subsequent *in vitro* study.

2.4. Cell adhesion

For the cell adhesion study, observation of cell morphology was conducted at day 1, 3 and 7 of cell seeding using FESEM. The cells were washed three times with PBS and treated with 10% neutral buffered formalin solution (Sigma-Aldrich) for 1 h. After fixation, the samples were subjected to dehydration in a graded series of ethanol with 30%, 50%, 70%, 90% and 100% concentration used for 10 min each, followed by freeze-drying (Labconco, USA) overnight.

2.5. Cell viability assay

The cell growth on control and nanopatterned samples was assessed by the alamar blue assay (resazurin reduction assay). A 10% working solution was prepared by diluting the resazurin stock solution (consisting of resazurin sodium salt powder (Sigma-Aldrich) in PBS with a concentration of 140 mg/L). After the prescribed culture period (day 1, 3 and 7), the old medium was discarded and the samples with seeded cells were rinsed 3 times with PBS. The samples were then transferred to a new 24-well plate (Nunc, Denmark) and 1 ml of working solution was added to each well. The samples with resazurin working solution were incubated for 4 h (at 34 °C under 5% CO_2 atmosphere). Wells only containing culture medium served as blank controls. After 4 h of incubation, a micropipette (Eppendorf, Germany) was used to transfer 100 µL triplicates of mixed solution from each well to a 96-well plate (TPP, Switzerland). The light absorbance value (optical density) was measured by a FLUOstar OPTIMA reader (BMC LABTECH) at 570 nm and 595 nm. The percentage of resazurin dye reduction was calculated by formula by the manufacturer's defined formula (Pettit et al., 2005). The assessment was carried out in darkness due to the light-sensitivity of the resazurin solution.

2.6. Alkaline phosphatase (ALP) activity

The ALP activity was obtained using Quantichrom ALP assay kit, DALP-250). At the prescribed culture period, samples were washed with PBS and then lysed with 0.5 mL of 0.2 Triton X. The well plate was shaken for 20 min at room temperature. The lysed cell lysate was centrifuged at 13,000 rpm for 5 min. 50 µL of the supernatant were transferred into 96-well plate and 150 µL of working solution were