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Microscopic observations of osteoblast growth on micro-arc oxidized β titanium

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ABSTRACT

Titanium alloys are widely used in orthopedic and dental implants, owing to their excellent physical properties and biocompatibility. By using the micro-arc oxidation (MAO), we generated anatase-rich (A-TiO_2) and rutile-rich (R-TiO_2) titanium dioxide coatings, individually on β-Ti alloy, in which the latter achieved an enhanced *in vitro* and *in vivo* performance. Thoroughly elucidating how the osteoblasts interact with TiO_2 coatings is of worthwhile interest. This study adopts the focused ion beam (FIB) to section off the TiO_2 coated samples for further scanning electron microscope (SEM) and transmission electron microscope (TEM) observation. The detailed crystal structures of the TiO_2 coated specimens are also characterized. Experimental results indicate osteoblasts adhered more tenaciously and grew conformably with more lamellipodia extent on the R-TiO_2 specimen than on the A-TiO_2 and raw β-Ti specimens. FIB/SEM cross-sectional images of the cell/TiO_2 interface revealed micro gaps between the cell membrane and contact surface of A-TiO_2 specimen, while it was not found on the R-TiO_2 specimen. Additionally, the number of adhered and proliferated cells on the R-TiO_2 specimen was visually greater than the others. Closely examining EDS line scans and elemental mappings of the FIB/TEM cross-sectional images of the cell/TiO_2 interface reveals both the cell body and interior space of the TiO_2 coating contain nitrogen and sulfur (the biological elements in cell). This finding supports the assumption that osteoblast can grow into the porous structure of TiO_2 coatings and demonstrating that the R-TiO_2 coating formed by MAO serves the best for β-Ti alloys as orthopedic and dental implants.

1. Introduction

Characterized by their high strength to weight ratio, corrosion resistance and satisfactory biocompatibility with living body, titanium alloys are widely used in implants for dental and orthopedic applications [1–5]. Naturally grown, dense and stable TiO_2 on titanium alloy surface has excellent biocompatibility. However, the host response to the naturally grown oxidized layer is not always favorable as a fibrous layer can form at the bone–device interface, incurring implant failure [6,7]. In addition to conventionally adopted methods such as sandblasting, acid-etching and plasma spraying of hydroxyapatite (HAp), numerous surface modification procedures [8] have been applied to enhance biological outcomes, including picosecond laser micromachining [9,10], fluoride ion modification [11], biomimetic modification [12,13] and plasma spraying using various materials [14,15]. However, these methods have one or some of such drawbacks as a low manufacturing throughout, toxic chemical solutions usage during processing, cost ineffectiveness and most importantly, unreliably weak coating/substrate adhesion during service. In contrast, micro-arc oxidation (MAO, or plasma electrolytic oxidation, PEO) procedure, in which an anodic voltage is applied onto the titanium metal in an electrolyte bath in order to produce agitated oxidation over the metal surface *via* the generated arc sparks (commonly referred to as "micro-arc"), has several advantages, including simple processing equipment, no limitations on the work piece size and shape, high growth rate of the oxidized layer and tailorable crystal structure by manipulating process parameters. Additionally, the as-grown MAO porous film can simultaneously achieve strong film/substrate adhesion due to the inward growth of the oxide layer and provide excellent osseous integration as well as biological fixation owing to the bone in-growth [16–18]. Therefore, MAO is potentially considered a superior surface modification method for biomedical implants and has a high biocompatibility for bone growth [16–19].

Owing to the above merits of the MAO procedure, a tremendous amount of effort has been paid to modify an osteo-compatible surface for titanium metal, with many of the obtained surface

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Table 1
Micro-arc oxidation parameters used in this study.

Parameters	Values
Electrolyte composition	0.05 M NaH_2PO_4
Applied voltage (V)	350 (to obtain A-TiO_2) and 450 (to obtain R-TiO_2)
Oxidation time (min)	20
Anode material (substrate)	CA-Ti (Ti–13Cr–3Al–1Fe, 1 cm × 1 cm × 0.1 cm)
Cathode material	Stainless steel (4 cm × 9 cm)
Bath temperature (°C)	25
Bath stirring	Magnetic stirrer

layer materials involving TiO_2 (unintentionally containing Ca and P) [20–22], TiO_2/TCP [23] and TiO_2/HAp [24], or even Sr-HAp [25]. However, the elastic modulus of pure titanium (E = 100 GPa) [26] and dual-phase titanium alloy (E = 114 GPa) [27] markedly exceed that of the human skeleton (E = 18 GPa) [26]. A high elastic modulus can lead to the stress shielding effect [28]. Conversely, owing to its high strength, low elastic modulus (E = 85–90 GPa), excellent corrosion resistance, and its vanadium-free nature [29], β-Ti alloy is widely regarded as a next-generation medical implant material [30,31]. Correspondingly, a recent study demonstrated the effectiveness of MAO treatment on β-Ti alloy to improve its biological performance [32]. Our previous studies focused on controlling the structure of the crystal phase to obtain anatase-rich titanium dioxide (A-TiO_2) and rutile-rich titanium dioxide (R-TiO_2) on β-Ti alloy surfaces [16,33], respectively. R-TiO_2 obviously has a higher *in vitro* osteoblast cell compatibility and improved *in vivo* osteogenesis performance over that of raw β-Ti alloy and A-TiO_2 [34,35]. This is in contrast with the results of Wu, which demonstrated the bioactive independency of the fraction of anatase and rutile over metal titanium surface to induce deposition of apatite in Kokubo's simulated body fluid [32].

The above studies generally assume that the oxide layer induces bone cell adhesion at the early stage. Therefore, microscopically understanding how the osteoblasts interact with TiO_2 coatings, particularly with different crystal structures, is of worthwhile interest. For microscopic observations, this study examines the feasibility of accurately sectioning off the MAO treated samples in specific areas where osteoblasts were previously cultured by using a focused dual-ion beam equipped with high-resolution scanning electron microscope (FIB/SEM). This FIB/SEM procedure can pinpoint the locations of interest where a viable osteoblast remains on the MAO treated surface. The sectioned-off cavity wall provides an ideal view for SEM cross-sectional observations and elemental composition analysis on both cultured osteoblast and MAO treated layer. In contrast with the commonly adopted top view for biological SEM observation, this study facilitates a successful lateral observation of the cell grown on MAO treated surface. Additionally, the sectioned samples are further analyzed by transmission electron microscope (TEM) to identify the crystal structure of MAO-TiO_2 and energy dispersive spectrometry (EDS) for identifying the biological elements in osteoblasts grown onto and into the MAO layer, revealing how MAO-TiO_2, in the form of A-TiO_2 and R-TiO_2, enhances the osteoblast growth.

2. Materials and methods

2.1. Specimen preparation

A Ti–13Cr–3Al–1Fe alloy, CA-Ti, purchased from Japan Daido Steel (Japan), was machined into a specimen sized 1.0 cm × 1.0 cm × 0.1 cm for MAO treatment, *in vitro* testing and microstructure characterization. Each specimen was ground, polished on one side by using SiC abrasive paper in sequence from #200 to #1200, and ultrasonically cleaned before MAO. Table 1 lists the MAO parameters in this study. Based on applied voltages of 350 V and 450 V, MAO layers with A-TiO_2 and R-TiO_2 were obtained separately, and the prepared specimens were denoted as A-TiO_2 and R-TiO_2, respectively. The crystal structures of MAO-treated specimens were then identified using a Bruker-D8 X-ray diffractometer (XRD) that applied Cu Kα (λ = 1.5405 nm) radiation. Next, the cross-sectional morphology and film thickness were determined using cold-field emission scanning electron microscopy (FESEM) (Hitachi S-4800). Additionally, the raw β-Ti alloy was used as a control sample in subsequent tests and analyses.

2.2. In vitro osteoblast activity of raw β-Ti alloy and MAO-treated specimens

Cell adhesion, cell proliferation, alkaline phosphatase (ALP) activity, osteopontin (OPN), osteocalcine (OCN), and calcium contents were determined, respectively, during the *in vitro* tests. The murine pre-osteoblast cell line MC3T3-E1 was obtained from the Riken Cell Bank (Tsukuba, Japan). In the cell adhesion assay, labeled MC3T3-E1 cells were plated on test specimens at a density of 10^4 cells/cm^2 and incubated for 2 h at 37 °C to facilitate adhesion. After washing twice with phosphate-buffered saline (PBS), non-adhering cells were removed by aspiration and, then, test specimens (with adhered cells on them) were read with a CytoFluor 2300 fluorescence plate reader (Millipore, MA, USA) to determine fluorescence intensity as an indicator of cell adhesion. Next, cell proliferation was assessed by measuring cell viability through use of the 3-[4,5-dimethylthiahiazo-2-yl]-2,5-diphenytetrazolium bromide (MTT) assay. MC3T3-E1 cells were cultured for 48 h on specimens at a density of 10^4 cells/cm^2. Optical absorbance at 550 nm was used as an indicator of cell proliferation and determined using a microplate reader (Bio-Tek, Winooski, VT, USA). Additionally, the ALP activity was evaluated using an ALP assay kit (Sigma, CA, USA). The MC3T3-E1 cells were cultured on test specimens (10^4 cells/cm^2) for 2 days. Optical absorbance level of supernatant aliquots subjected to a protein assay using the Pierce Coomassie Plus assay reagent (Pierce, Rockford, IL, USA) was read at 405 nm (Bio-Tek, Winooski, VT, USA) to determine ALP activity as an indicator of cell differentiation.

Owing to that OPN and OCN are important markers at the late stage of osteoblast differentiation, this study evaluated osteogenesis performance by assessing OPN, OCN and calcium contents to determine osteogenesis performance. The MC3T3-E1 cells were cultured on test specimens and incubated for 48 h at 37 °C. The OPN and OCN contents were assayed using OPN and OCN enzyme immunoassay kits, following the manufacturer's instructions (Biocompare, San Jose, CA, USA). Calcium content was then determined by assessing the mineralized nodule formation. Next, the MC3T3-E1 cells (pre-osteoblast) were cultured in α-minimal essential medium (α-MEM) containing 50 μg/ml vitamin C and 10 mM β-glycerophosphate. Additionally bone nodule formation was determined on day 10 using alizarin red-S staining. Finally, the alizarin red-S content in the test samples was quantified by measuring optical absorbance at 550 nm and calculated according to a standard curve.

2.3. Microscopic observation of osteoblast cultured on raw β-Ti alloy and MAO-treated specimens

This study investigated the surface and cross-sectional morphology of osteoblast growth on raw β-Ti alloy, A-TiO_2, and R-TiO_2 specimens by culturing the MC3T3-E1 cells (pre-osteoblast) on each specimen for 2 and 48 h. Cells grown on each specimen were fixed with sodium phosphate-buffered glutaraldehyde (2.5%, pH 7). After washing twice in the same buffer, cells were dehydrated with graded ethanol at room temperature. All