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Interface between grown osteoblast and micro-arc oxidized bioactive layers

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ABSTRACT

To enhance the osseointegration of titanium implants, bioactive ceramic layers are grown on titanium substrate; they combine the osteoconductivity of hydroxyapatite (HAp) with the biological activity of TiO_2. However, the effects of the adhesion and growth behavior of osteoblasts on bioactive ceramic coatings remain to be elucidated. Anatase (A-TiO_2), rutile (R-TiO_2), HAp and HAp-TiO_2 dual-phase coatings were fabricated by micro-arc oxidation (MAO) on a titanium plate. The mechanism by which bioactive MAO layers induced cell growth at the interfaces between the coatings and the growing osteoblasts was investigated. Experimental results indicate that the bioactive ceramic coatings that were formed by MAO supported much greater cell mineralization than those formed on bare Ti. Among the four types of MAO coatings, the HAp-TiO_2 coating provided the best cell mineralization owing to its abundance of Ca^{2+} ions and OH^- groups, which promoted the formation of calcium phosphate, and consequently, cell attachment and growth. Surface and interfacial microscopic observations revealed that, at the beginning of the cell culture test, osteoblasts attached strongly to the inner walls of the pores in the HAp-TiO_2 coating. Moreover, osteoblasts on HAp and HAp-TiO_2 coatings formed a large extracellular matrix (ECM) after 7 days. Over 21 days, acicular and coral-shaped bone-like apatite structures were found to be formed by the osteoblasts that were cultured on both the HAp and the HAp-TiO_2 coatings; the cells were attached tightly to these coatings and to the inner wall of their pores. TEM observations verified that both the acicular and the coral-shaped bone-like apatite structures were hydroxyapatite.

1. Introduction

Titanium (Ti) and its alloys have excellent biocompatibility, corrosion resistance, and mechanical properties and so are extensively used in dental and orthopedic implants. However, the bio-inertness and ion release of Ti-based implants may inhibit their direct bonding with bone tissue during implantation [1,2]. Hence, efforts have been made to strengthen the bonding between implant and bone tissue to enhance osseointegration and reduce the healing period.

Hydroxyapatite (HAp, $Ca_{10}(PO_4)_6(OH)_2$) is a bioactive ceramic material with excellent biocompatibility and osteoconductivity [3], so it easily forms calcium phosphate that is strongly chemically bonded to the surface of an implant [4]. This strong chemical bond facilitates initial implant stabilization and osseointegration further accelerates repair of the damaged bone tissue. However, the biodegradability and poor intrinsic mechanical properties of HAp result in the high failure rate of implanted prosthetics. A native titanium dioxide (TiO_2) layer forms on the surface of titanium upon contact with air or water [5]. The TiO_2 layer is well known for its density, non-cytotoxicity [6], favorable catalytic activity [7], and highly antibacterial properties [6,8]. Furthermore, the free radicals on a TiO_2 surface can absorb the Ca^{2+} and PO_4^{3-} ions in proteins, forming apatite that subsequently induces osteoblast adhesion [9]. Bioactive ceramic composite coatings, especially those that combine HAp and TiO_2, have been extensively studied [10–14] to improve both the attachment between HAp coating and bone cells and their biocompatibility on the surface of titanium.

Micro-arc oxidation (MAO) is a relatively convenient and effective technique for fabricating porous ceramic coatings on metal surfaces and an MAO-modified film adheres strongly to the implants applied to titanium substrates. Therefore, the MAO is regarded as a superior method of surface modification for biomedical implants. Many investigations have shown that the osteoconductivity of implants depends significantly on their surface properties [15], such as their chemical composition [16–18], surface roughness [19,20], morphology [21], and cellular interaction. The interactions between osteoblasts and various MAO-modified ceramic coatings were examined microscopically in this study. Osseointegration between the osteoblast and anatase

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

Parameter		Value			
		A-TiO_2	R-TiO_2	HAp	HAp-TiO_2
Ca/P ratio		1.00	1.10	2.16	1.66
Bath pH value		4.81	12.00	5.04	4.94
Electrolyte (M)	$Ca(CH_3COO)_2\ H_2O$	0.12	0.13	0.26	0.20
	$NaH_2PO_4\ 2H_2O$	0.12	0.12	0.12	0.12
Applied voltage (V)		430	450	450	480
Oxidizing time (min)		5	5	10	10

(A-TiO_2), rutile (R-TiO_2), hydroxyapatite (HAp), and dual-phase hydroxyapatite-titanium dioxide (HAp-TiO_2) coatings, fabricated by MAO, was investigated. The osteoblast mineralization test on bare Ti and MAO-modified coatings revealed the mineralized situation between the coatings and the growing osteoblasts, and a dual-beam focused ion beam system (DB-FIB) was utilized to elucidate how the bioactive MAO layers affect cell growth. Energy dispersive spectrometry (EDS) was utilized to identify the biological elements in osteoblasts that were grown onto and into MAO coatings.

2. Experimental

2.1. Preparation of specimen

Grade II pure titanium (CP Ti) metal was machined into a 20 mm × 20 mm × 1 mm thin plate. Table 1 presents the MAO parameters that were used in this study. Various calcium acetate ($Ca(CH_3COO)_2\ H_2O$) concentrations with a fixed concentration of sodium phosphate dibasic dehydrate ($NaH_2PO_4\ 2H_2O$) were employed to obtain various ratios of calcium and phosphorus, and various pH values, applied voltages, and oxidation times were used in the MAO process to prepare the target phase structure. The crystal structures of MAO-treated coatings were identified using a Bruker-D8 X-ray diffractometer (XRD) with Cu Kα (λ = 1.5405 nm) radiation. The cross-sectional morphology and the thickness of each film were determined using a cold-field emission scanning electron microscope (FESEM). A X-ray photoelectron spectroscope (XPS, ESCA PHI 1600, USA) with Mg Kα (1253.6 eV) radiation was utilized to determine the compositions of the surface.

2.2. Cell culture and microstructural observation

A murine pre-osteoblast cell line (MC3T3-E1), obtained from the RIKEN Cell Bank (Tsukuba, Japan), was cultured three times ($N = 3$) on bare Ti and MAO-modified coatings for 21 days to observe three times ($N = 3$) the conditions of cell mineralization [22]. To quantify the calcification of a matrix by the cultured osteoblasts, Alizarin Red S binding assay was used. After 21 days of culturing the osteoblasts, the medium was removed, 40 mM Alizarin Red S [0.5% (vol/vol) Alizarin Red S, pH 4.2] was added, and the system, containing the osteoblasts, was incubated at room temperature for 10 min. The plates were washed three times in PBS before 0.1 ml of 10% (wt/vol) cetylpyridinium chloride was added for 10 min to release the remaining calcium-bound Alizarin Red S. The solution was collected and measured at OD550 on an ELISA reader.

Osteoblasts were cultured for 1, 7 and 21 days, and the cells were observed. At each time point, the medium was removed, the cells were fixed with 2.5% glutaraldehyde solution for 20 min, and two-step dehydrations (serial alcohol dehydration and critical point drying (CPD)) were performed. All samples were sputtered with an Au film at 20 mA for 3 min to improve the quality of the observations. Cellular topographies, cross-sectional images, and TEM samples were obtained using an FEI Helios 600i dual beam focused ion beam (DB-FIB). The stage was initially tilted to 52° (perpendicular to FIB) and the protective Pt layer (10 μm × 2 μm × 1.5 μm) that was deposited on the surface of cells. Then, regular cross-sectional milling (to yield a sample with a thickness of 1.5 μm) at 9.3 nA and cleaning cross-sectional milling (to yield a sample thickness with a thickness of 800 nm) at 80 pA were carried out. Energy dispersive spectrometers (EDSs) were used to obtain profiles of the osteoblasts, MAO-modified coatings, and Ti substrate to elucidate the cell growth behavior. Finally, the milled sample was formed into TEM foil (<80 nm thick) to make field emission gun transmission electron microscopic (FEG-TEM) observations of their microstructures.

3. Results and discussion

3.1. Microstructural characterization

Fig. 1 presents the XRD diffraction patterns and SEM morphologies of A-TiO_2, R-TiO_2, HAp, and dual-phase HAp-TiO_2 coatings that were produced by MAO [23]. Changing the Ca/P ratio, the applied voltage and the oxidizing time produced different phase structures. XPS was utilized to investigate the chemical composition of these four MAO coatings, yielding the elemental concentrations at the surface that are presented in Table 2. The MAO coatings contain mostly Ti, O, Ca, and P (Fig. 2(a)). The binding energies (BE) for Ti 2p3/2 and Ti 2p1/2 are 460.7 ± 0.2 eV and 466.2 ± 0.2 eV (Fig. 2(b)), respectively. These peak positions correspond to a chemical state of Ti^{4+} [24] and are

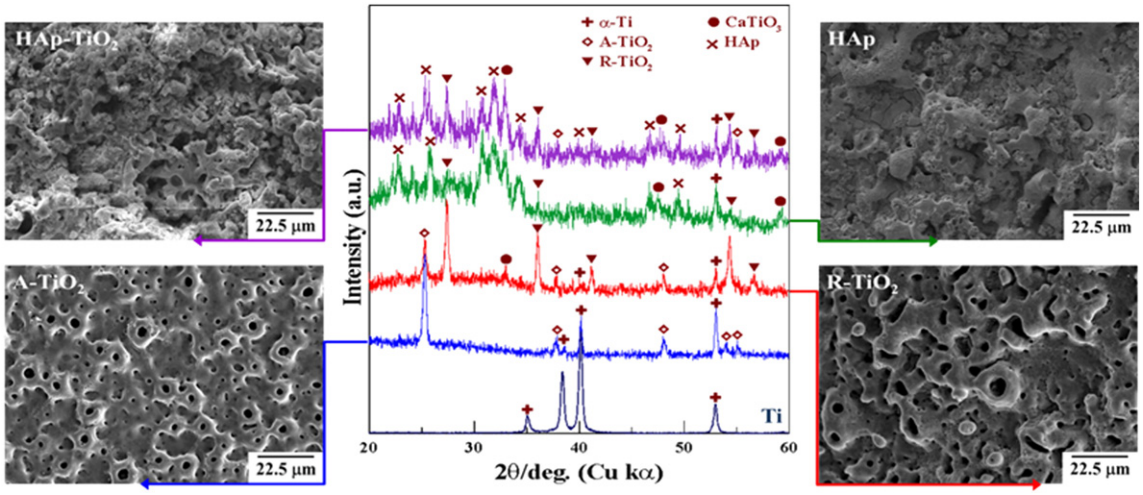

Fig. 1. XRD pattern and SEM-determined morphology of MAO coatings.