Materials Science and Engineering C 49 (2015) 7–13

Contents lists available at ScienceDirect

Materials Science and Engineering C

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

Characterization of the aspects of osteoprogenitor cell interactions with physical tetracalcium phosphate anchorage on titanium implant surfaces

Chia-Ling Ko [a,b], Ya-Yuan Chang [c], Cian-Hua Liou [c], Wen-Cheng Chen [a,*]

[a] Advanced Medical Devices and Composites Laboratory, Department of Fiber and Composite Materials, Feng Chia University, Taichung 40724, Taiwan
[b] Dental Medical Devices and Materials Research Center, College of Dental Medicine, Kaohsiung Medical University, Kaohsiung 807, Taiwan
[c] Alliance Global Technology Co., Ltd., Kaohsiung Medical Device Special Zone in Southern Taiwan Science Park, Kaohsiung 82151, Taiwan

ARTICLE INFO

Article history:
Received 12 June 2014
Received in revised form 29 August 2014
Accepted 17 December 2014
Available online 19 December 2014

Keywords:
Tetracalcium phosphate
Surface modification
Progenitor cell
Implant
Osseointegration

ABSTRACT

Well-designed implants are used not only to modify the geometry of the implant but also to change the chemical properties of its surfaces. The present study aims to assess the biofunctional effects of tetracalcium phosphate (TTCP) particles as a physical anchor on the implant surface derived through sandblasting. The characteristics of the surface, cell viability, and alkaline phosphatase (ALP) activity toward osteoprogenitor cells (D1) were obtained. D1 cells were cultured on a plain surface that underwent sandblasting and acid etching (SLA) (control SLA group) and on different SLA surfaces with different anchoring TTCP rates (new test groups, M and H). The mean anchoring rates were 57% (M) and 74% (H), and the anchored thickness was estimated to range from 12.6 μm to 18.3 μm. Compared with the control SLA surface on Ti substrate, the new test groups with different TTCP anchoring rates (M and H) failed to improve cell proliferation significantly but had a well-differentiated D1 cell phenotype that enhanced ALP expression in the early stage of cell cultures, specifically, at day 7. Results suggest that the SLA surface with anchored TTCP can accelerate progenitor bone cell mineralization. This study shows the potential clinical application of the constructed geometry in TTCP anchorage on Ti for dental implant surface modification.

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1. Introduction

Titanium (Ti) is a well-known implant biomaterial for clinical application because of its ideal mechanical properties, excellent biocompatibility, long-term stability, and safety [1]. Branemark et al. utilized commercially pure Ti implants in animals and demonstrated direct contact of Ti with bone [2]. Such biological modification in which direct structural and functional connection between living bone and surfaces of an implant is present is called osseointegration. In the last decade, many researchers have attempted to improve the efficiency of osseointegration in shortening the bone-healing process because the clinical success of oral implants is related to early osseointegration [3]. These researchers confirmed that surface properties including geometry, topography, roughness, and chemistry are important factors of osseointegration efficiency [3–7]. Numerous modifications, such as sandblasting, acid etching, plasma spraying, and anodization, have been applied in manufacturing processes to fabricate the various surfaces of Ti implants [5–13]. Among these surface modifications, the simplest, most popular, and most cost-effective means is to combine sandblasting and acid etching (SLA) and create a topography with 3D macro-sized and micro-sized geometry. Most dental implants are moderately rough (Ra ranging within 1.0–2.0 μm) [5] to induce protein adhesion, cell attachment, cell proliferation, and cell differentiation and to support mechanical stability [3,8–13]. High-precision control of roughness through SLA has been proven to enhance the rate and degree of osseointegration in animal studies and subsequent histomorphometric evaluations [14–16]. However, further development of implants not only attempts to modify the geometric design but also to change the chemical properties of implant surfaces, such as changing the chemical composition, ameliorating hydrophilicity, and tethering growth factors on surfaces [11–14,17,18]. Surface modification can mimic the organic and inorganic components of bone tissues and accelerate physiological transition layer establishment between living bones and implant surfaces [19,20]. A typical example is applying calcium phosphates (CaPs) on implant surface through coating, plasma-based ion implantation, or physical deposition to provide numerous Ca and phosphate ions that enhance protein adhesion and biological performance [19–23]. Among CaPs, tetracalcium phosphate (TTCP) is the only compound with a Ca/P ratio of 2.0, which is greater than that of stoichiometric hydroxyapatite (1.67) [24]. When TTCP dissolves in the body fluid, it can release large amounts of ions of Ca^{2+} and PO_4^{3-}. The degradation of TTCP and its transformation into apatite known from experiments in neutrally buffered aqueous solutions [25] supported early bone formation and led

* Corresponding author at: Advanced Medical Devices and Composites Laboratory, Department of Fiber and Composite Materials, Feng Chia University, 100, Wenhwa Rd., Seatwen, Taichung 40724, Taiwan, ROC.
E-mail addresses: wencchen@fcu.edu.tw, wincheng0925@yahoo.com.tw (W.-C. Chen).

http://dx.doi.org/10.1016/j.msec.2014.12.063
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to optimal gap/interface healing [24,25]. Moreover, TTCP-derived calcium phosphate cement (CPC) has been proven to be almost completely resorbed 24 weeks after implantation in rabbit femur and that bone remodeling is almost finished at the cortical site after the same period [26]. Considering that CaPs cannot bond naturally on a Ti surface, many different surface treatment strategies have been developed to attach CaPs on the implant surface. These strategies include physical anchorage through sandblasting, plasma spraying, sputter deposition, sol–gel coating, electrophoretic deposition, and precipitation [27]. The aim of this study is to assess the physical–chemical properties and investigate the biofunctional effects of TTCP as a physical anchor on the implant surface through sandblasting.

2. Materials and methods

2.1. Preparation of Ti disks and implants

Commercially pure, grade IV, cylindrical Ti (ASTM 67) was cut by computer numerical control to 3.5 mm diameter × 8 mm length for integration tests. The controlled standard Ti surface with a mean Ra value of 0.07 μm within 10.0% standard deviation (SD) was prepared before the SLA process. The surfaces were then sandblasted with alumina (Al_2O_3) particles with particle size ranging from 150 to 250 μm using an air compressor. After sandblasting, the samples were acid-etched to fabricate surfaces for SLA group. The etching solution contained HCl (37%), H_2SO_4 (96%), and H_2O in a volume ratio of 1:1:1. Prior to the second surface treatment process, all specimens were washed twice with ethanol and distilled water through ultrasonic oscillation.

2.2. TTCP fabrication

TTCP powder was prepared through a reaction suggested by Brown and Epstein [22]. The reaction is as follows:

$$2CaCO_3 + Ca_2P_2O_7 \rightarrow Ca_4P_2O_9 + 2CO_2. \quad (1)$$

A mixture of 400 g of $CaCO_3$ (Shimakyu's Pure Chemicals, Osaka, Japan) and 508 g of $Ca_2P_2O_7$ (Alfa Aesar, Johnson Matthey Company, MA, USA) was briefly mixed with 750 mL ethanol (99.8%). The resultant slurry was mixed uniformly and dried in an oven at 60 °C. The dried mixture was then heated at 1400 °C for 12 h, immediately cooled at room temperature, after which it was ground and sieved with a 325-mesh screen. The final mean particle size of TTCP was 10.1 ± 0.7 μm.

After SLA treatment and cleaning, the experimental specimens were sandblasted with TTCP particles using an air compressor with 4–6 kg/m^2 powder blast over a 3 cm distance for 10 s and marked as moderate capping area group of anchored TTCP (denoted as M). Another set underwent the same process but was blasted for 15 s and marked as the high capping area group of anchored TTCP (denoted as H). The new test groups were cleaned using an ultrasonicator at 28 kHz that lasted at least 1 h. Then, the samples were rinsed and dried.

2.3. Surface characterizations

The topography of the different groups was obtained using a scanning electron microscope (SEM, Hitachi S-3000N, Japan) equipped with an energy dispersive spectroscopy (EDS, Horiba EX220, Japan) system to analyze TTCP-anchored/residual areas on the surface. Image J software version 1.34s (NIH, Bethesda, MD, USA) was utilized to measure TTCP anchored/residual area.

Surface phase analysis was conducted using an X-ray diffractometer (XRD-6000, Shimadzu, Japan) operating at 40 kV and 30 mA with Ni-filtered Cu Kα radiation. The low-angle X-ray diffraction mode with incident angle ω, which is defined as the angle between the X-ray source and the sample, varied in 1°, 2°, 3°, 5°, 10°, 12°, 15°, 20°, and 25°. Standard low-angle X-ray diffraction 2θ–θ mode can reveal different types of peak profiles and has a depth-profiling relationship. A low incident angle indicates a scattering ray by detected phase diffractions and that depth profile is close to the surfaces. The depth of the X-ray penetration can be calculated to estimate the approximate thickness of anchored TTCP. Depth in this study was derived by the following equation:

$$E = h \times C/\lambda = h \times \nu \quad (2)$$

where h is the constant 6.626×10^{-34} J s, ν is the frequency, λ is the wavelength, and E is the incident energy. Eq. (2) was then simplified into Eq. (3), where the incident depth is proportional to diffraction E and the incident angles are as follows [28–30]:

$$E = \sin \omega/\mu \quad (3)$$

where μ represents the linear absorption coefficient and the values of 1/μ are generally within the range of 10 μm to 100 μm. When the energy was 8.1×10^{-3} MeV, the linear absorption coefficient of Ti was 202.30 cm^2/g. The X-ray was operated with a scan angle range of 2θ from 10° to 80° and a scan rate of 2.0°/min. Phases were identified by matching each characteristic peak with those of the Joint Committee on Powder Diffraction Standards (JCPDS) files.

2.4. Cell cytotoxicity

The cytotoxic effects of Ti implants subjected to different surface treatments on NIH-3T3 were tested following the standard of ISO 10993-5 with a commercially available proliferation kit (XTT assay, Biological Industries, Israel). The extraction solution was prepared at the ratio of 1:5 (1 g sample per 5 mL medium). The implants were immersed in a culture medium for 24 h. The cells were seeded in 96-well culture plates at a density of 1×10^4 cells per well in Dulbecco's modified Eagle's medium (DMEM) and allowed to attach for 24 h. DMEM was then removed, and the Ti implant extraction solution was added to the 96-well culture plates. The negative control sample was treated with Al_2O_3 powder culture medium, the positive control was mixed with 15% dimethyl sulfoxide (DMSO) culture medium, and the control was placed in an untreated culture medium. After 24 h of culture, 50 μL of XTT reaction solution (sodium 3′-[1-(phenyl-aminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzenesulfonic acid hydrate and *N*-methyl dibenzopyrazine methyl sulfate, 50:1) was added to the wells. Optical density was recorded at a wavelength of 492 nm using an ELISA plate reader EZ Read 400 (Biochrom, UK) after incubating the plates using XTT (at 37 °C and with 5% CO_2 + 95% air) for 4 h. Triplicate experiments were performed.

2.5. Cell–surface interactions

Bone marrow mesenchymal stem cells (osteoprogenitor cells; D1) cloned from Balb/C mice were purchased from American Type Culture Collection. D1 cells were maintained in DMEM supplemented with 10% fetal bovine serum in 37 °C incubators with 5% CO_2. After a confluent cell layer was formed, the cells were detached by submerging to 0.25% trypsin in phosphate buffered saline (PBS) and resuspended in DMEM. Cells were utilized before the eighth passage.

The different Ti implants were placed into Eppendorf tubes where 50 μL containing 5×10^5 cells was dripped onto the implants for 1 h. After 1 h, the implants and the cell suspension medium were transferred into 96-well ultra-low attachment surface plates. Then, 50 μL of culture medium was added to swamp the implants with culture. Incubation periods were set at 1 h, 1 d, and 2 d. PBS was utilized to wash the substrates carefully at different periods, and then the cells were fixed with glutaraldehyde. The implants were then gold-plated and screened through SEM.

Cell viability at time points of 1, 4, 7, 10, 14, and 18 d was determined after an initial seeding of 1×10^5 D1 cells on the surface. The culture

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