Applied Surface Science 329 (2015) 347–355

Contents lists available at ScienceDirect

Applied Surface Science

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

Physicochemical properties and *in vitro* cytocompatibility of modified titanium surfaces prepared via micro-arc oxidation with different calcium concentrations

Sui-Dan Wu[a], Hui Zhang[a], Xu-Dong Dong[b], Cheng-Yun Ning[c], Alex S.L. Fok[d], Yan Wang[a,*]

[a] Guanghua School of Stomatology, Hospital of Stomatology, Guangdong Key Laboratory of Stomatology, Sun Yat-sen University, Guangzhou 510055, China
[b] Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, BC V6T 1Z3, Canada
[c] College of Material Science and Engineering, South China University of Technology, Guangzhou 510641, China
[d] Minnesota Dental Research Center of Biomaterials and Biomechanics, School of Dentistry, University of Minnesota, Minneapolis, MN 55414, USA

ARTICLE INFO

Article history:
Received 30 June 2014
Received in revised form 24 October 2014
Accepted 7 December 2014
Available online 16 December 2014

Keywords:
Micro-arc oxidation
Titanium
Surface modification
Cytocompatibility
Calcium concentration

ABSTRACT

Objective: To explore the effect of calcium concentration in the electrolyte solution on the physicochemical properties and biocompatibility of coatings formed by micro-arc oxidation (MAO) on titanium surfaces.
Methods: The surfaces of pure titanium plates were modified by MAO in an electrolytic solution containing calcium acetate (CA; $C_4H_6CaO_4$) at concentrations of 0.05, 0.1, 0.2, or 0.3 M and β-glycerophosphate disodium salt pentahydrate (β-GP; $C_3H_7Na_2O_6P{\cdot}5H_2O$) at a fixed concentration of 0.02 M. Surface topography, elemental characteristics, phase composition, and roughness were investigated by scanning electron microscopy, energy-dispersive X-ray analysis, X-ray diffraction, and a surface roughness tester, respectively. To assess the cytocompatibility and osteoinductivity of the surfaces, MC3T3-E1 pre-osteoblasts were cultured on the surfaces *in vitro*, and cell morphology, adhesion, proliferation, and differentiation were observed.
Results: The porous MAO coating was composed primarily of TiO_2 rutile and anatase. The amount of TiO_2 rutile, the Ca/P ratio, and the surface roughness of the MAO coating increased with increasing CA concentration in the electrolyte solution. $Ca_3(PO_4)_2$, $CaCO_3$, and $CaTiO_3$ were formed on MAO-treated surfaces prepared with CA concentrations of 0.2 and 0.3 M. Cell proliferation and differentiation increased with increasing CA concentration, with MC3T3-E1 cells exhibiting favorable morphologies for bone–implant integration.
Conclusions: MAO coating improves the surface characteristics and cytocompatibility of titanium for osseointegration. Higher CA concentration in the MAO electrolyte solution has a positive effect on the surface properties, chemical composition, and cell response.

1. Introduction

Titanium and titanium-based alloys have been widely used in orthopedic and oral implants due to their favorable biocompatibility, corrosion resistance, and low elastic modulus. When these materials are exposed to air, an oxide layer spontaneously forms on the titanium surface, which can lead to poor initial osseointegration and eventual implant failure, because the bioinert oxide layer does not promote osteoblast attachment and growth [1,2]. Therefore, various surface modification techniques have been applied to improve the cytocompatibility of titanium implant surfaces in order to support osseointegration of implants with surrounding tissues as well as to reduce the risk of metal ion leakage [3]. These include mechanical-physical methods such as sandblasting, plasma spraying, and ion sputtering as well as chemical methods such as etching, biomimetic synthesis, and the sol-gel method. Among these techniques, sandblasting, acid-etching, and plasma spraying are the most commonly used techniques at present. Through sandblasting and acid-etching, a rough surface can be formed on the titanium implant surface, which facilitates the attachment and growth of osteoblasts. However, the titanium surface produced remains inert. With plasma spraying, bioactive materials can be sprayed onto the titanium surface to form a coating [4]. However, coatings produced using this technique that

* Corresponding author. Tel.: +86 2083802805.
E-mail addresses: wyan65@163.com, wangyan9@mail.sysu.edu.cn (Y. Wang).

http://dx.doi.org/10.1016/j.apsusc.2014.12.039
0169-4332/

involves a high temperature tend to delaminate due to stress that occurs during the cooling process [5,6]. Therefore, new surface modification techniques continue to be a major research focus.

Micro-arc oxidation (MAO) is another surface treatment method that applies an electrochemical process to generate an oxide coating on metals. In this process, ions in an electrolyte solution are incorporated with the substrate metal ions by instant local high potential, temperature, and pressure, producing a new membrane in situ on top of the metal substrate and modifying the structure of the previous oxide layer [7]. The newly formed coating adheres strongly to the metal substrate and shows high resistance to wear and corrosion. Compared with the acid-etching and plasma spraying approaches, the MAO technique offers the following advantages: bioactive elements in the electrolyte solution can be introduced into the coating; thermal stress can be greatly reduced via a gradient coating design; and the characteristics of the coating can be monitored by adjusting the electrical parameters and the composition of the electrolyte solution [8–11]. Therefore, MAO treatment has been applied to titanium surface modification, specifically in the development of dental implant materials.

As mentioned above, two critical factors control the characteristics of the MAO coating: the electrical parameters and electrolyte formulation. Currently, the most commonly used MAO electrolytes for dental titanium implant coating are calcium acetate (CA; $C_4H_6CaO_4$) and β-glycerol phosphate disodium salt pentahydrate (β-GP; $C_3H_7Na_2O_6P{\cdot}5H_2O$). Via the electrochemical reaction, calcium and phosphorus can be introduced into the coating [12]. It had widely been accepted that calcium ions play an important role in the process of bone growth and osseointegration [13]. Many studies have reported that bone cells show greater adherence, spreading, and growth ability on Ca^{2+} incorporating titanium surfaces than on nontreated titanium surfaces [14–16]. However, the effect of Ca^{2+} concentration of electrolyte on the surface properties and cytocompatibility of MAO coatings has not been well elucidated. In a few relevant studies, various electrolytes and working conditions were used to get different ion composition and concentration (Ca, S, P, etc.) on the MAO coating surface to observe the *in vitro* and *in vivo* bone cell response, but the results varied and the comparison was relatively difficult [16–19]. To straightforwardly compare the effect of the change of the electrolyte calcium ion concentration on the surface properties and cytocompatiblity, we keep the key electrolyte composition consistent and change their relative concentration, attempting to get a relatively broad range of Ca^{2+} concentration ingredient on the MAO coating surface. The purpose of this study aims to determine the effect of calcium concentration on the surface properties and cytocompatibility of titanium MAO coatings. The surface topography, elemental distribution, phase composition, and roughness of the MAO coatings were investigated by scanning electron microscopy (SEM), energy-dispersive X-ray analysis (EDS), X-ray diffraction (XRD), and a surface roughness tester, respectively. To assess the cytocompatibility and osteoinductivity of the MAO coatings, preosteoblasts were cultured on the modified titanium surfaces *in vitro*, and cell morphology, adhesion, proliferation, and differentiation were evaluated.

2. Materials and methods

2.1. MAO coating preparation

Pure titanium specimens of 10 mm in diameter and 2 mm in thickness were machine-cut from a pure titanium rod (Goodfellow, Cambridge, UK). Both the top and bottom surfaces were ground with #260 to #1000 SiC sandpaper successively; ultrasonically cleaned with acetone, anhydrous alcohol, and deionized water in sequence; and finally air dried at room temperature. Specimens used as blank controls were left untreated, and those in the four treatment groups were subjected to MAO processing in electrolyte solutions containing four different calcium concentrations (0.05, 0.1, 0.2 and 0.3 M). The MAO electrolyte solutions were prepared by dissolving CA monohydrate ($(CH_3COO)_2Ca{\cdot}H_2O$) and β-GP in deionized water. The concentration of β-GP was 0.02 M in all four test solutions. The discs were immersed in 1 L of electrolyte solution and processed using MAO equipment (Model 20, Pulsetech, Chengdu, China). The parameters of the MAO treatment were as follows: voltage of 420 V, frequency of 100 Hz, duty cycle of 30%, and duration of 5 min. After MAO treatment, the specimens were rinsed with distilled water and dried in a drying cabinet.

2.2. Characterization of MAO-treated titanium surfaces

The topography of pure titanium and MAO-treated surfaces was observed by SEM (LEO 1530 FESEM, Oxford Instruments, Oxford, UK). For the MAO coatings, three specimens for each group and five vision fields per specimen were used to measure and calculate the mean pore size with Image Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA). To observe the morphology of cross-sections of the MAO coatings, specimens were first embedded in 1:1 epoxy and polyamide resin, then polished to a mirror-like finish with SiC sandpaper, and finally prepared for SEM observation. The composition of the MAO coating was detected by EDS incorporated with SEM. The crystalline phase of the MAO coating was analyzed by XRD (Panalytical X'Pert Pro MPD, The Netherlands), and finally, the average roughness (Ra) of the surface was measured using a roughness tester (Accretech, Tokyo, Japan).

2.3. Cell culture

MC3T3-E1 cells (Shanghai Chinese Academy of Science, Shanghai, China) were incubated in α-Minimal Essential Media with glutamax (Gibco, Grand Island, NY, USA) and 10% fetal calf serum (Gibco) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. The cell culture medium was changed every 3 days. When the cells reached 80–90% confluency, a trypsin-EDTA solution (Gibco) was used to detach the cells from the culture flasks. Detached cells were then transferred to the titanium samples at a density of 2×10^4 cells/ml.

2.4. Cell morphology

MC3T3-E1 cells seeded onto the surfaces of treated and control titanium discs were cultured for 1 h, 4 h, or 2 days following standard procedures. At the end of the culture period, the samples were washed with phosphate-buffered saline (PBS), fixed with 2.5% glutaraldehyde buffered by PBS, dehydrated in a graded series of alcohol (30%, 50%, 70%, 90%, and 100%), and then dried in a drying cabinet. The dried samples were sputter-coated with gold, and cell morphology was observed by SEM to evaluate early adherence and proliferation.

2.5. Cell proliferation

The Cell Counting Kit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan) was used to evaluate the proliferation of cells cultured on the modified titanium surfaces. Cells seeded onto the surfaces of the treated and control titanium discs were incubated at 37 °C in 5% CO_2 for 1, 4, or 7 days. At each time point, the titanium discs were transferred to new 24-well plates and rinsed with PBS. Then, 45 μl CCK-8 solution and 455 μl Dulbecco's Modified Eagle Medium (DMEM, Gibco) without serum were added to each well. After incubation at 37 °C for 3 h, 100 μl supernatant from each well was transferred to wells of a 96-well plate. Absorbance was measured