Materials Science and Engineering C 53 (2015) 272–279

Contents lists available at ScienceDirect

Materials Science and Engineering C

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

Guided proliferation and bone-forming functionality on highly ordered large diameter TiO_2 nanotube arrays

Ruopeng Zhang, Hongliu Wu, Jiahua Ni *, Changli Zhao, Yifan Chen, Chengjunyi Zheng, Xiaonong Zhang *

State Key Laboratory of Metal Matrix Composites, School of Material Science and Engineering, Shanghai Jiao Tong University, Shanghai 200240, P R China

ARTICLE INFO

Article history:
Received 28 July 2014
Received in revised form 23 March 2015
Accepted 26 April 2015
Available online 29 April 2015

Keywords:
TiO_2 nanotube arrays
Large diameter
MC3T3-E1 cell
Proliferation rate
Cell morphology
ALP activity

ABSTRACT

The significantly enhanced osteoblast adhesion, proliferation and alkaline phosphatase (ALP) activity were observed on TiO_2 nanotube surface in recent studies in which the scale of nanotube diameter was restricted under 100 nm. In this paper, a series of highly ordered TiO_2 nanotube arrays with larger diameters ranging from 150 nm to 470 nm were fabricated via high voltage anodization. The behaviors of MC3T3-E1 cells in response to the diameter-controlled TiO_2 nanotubes were investigated. A contrast between the trend of proliferation and the trend of cell elongation was observed. The highest cell elongation (nearly 10:1) and the lowest cell number were observed on the TiO_2 nanotube arrays with 150 nm diameter. While, the lowest cell elongation and highest cell number were achieved on the TiO_2 nanotube arrays with 470 nm diameter. Furthermore, the ALP activity peaked on the 150 nm diameter TiO_2 nanotube arrays and decreased dramatically with the increase of nanotube diameter. Thus a narrow range of diameter (100–200 nm) that could induce the greatest bone-forming activity is determined. It is expected that more delicate design of orthopedic implant with regional abduction of cell proliferation or bone forming could be achieved by controlling the diameter of TiO_2 nanotubes.

1. Introduction

After the first introduction of highly ordered, vertical-oriented, anodized TiO_2 nanotube arrays associated with the efforts of Zwilling et al. [1], a wide range of applications based on its unique structure and properties has been explored [2–9]. Along with the refinement of anodization methods to fabricate TiO_2 nanotube arrays [10,11], its potential as orthopedic implant material and substrate of tissue engineering has attracted increasing attention due to the biocompatibility and the unique cellular structure [12,13].

It has been proven that cells could sense and respond to nanostructure in most published literatures [9,13–15]. The significantly enhanced cell adhesion, proliferation and altered differentiation behavior were observed on TiO_2 nanotube surface in recent studies [12,15,16]. Park et al. seeded mesenchymal stem cells on the amorphous TiO_2 nanotube arrays with serial diameters ranging from 15 nm to 100 nm. It was observed that the highest proliferation ratio appeared on the nanotube surface with 15 nm diameter, and significant cell apoptosis appeared on the nanotube surface with 100 nm diameter [17]. On the contrary, Brammer et al. observed a significantly different pattern of cell response of MC3T3 cells on the annealed TiO_2 nanotube arrays within the similar range of diameters. They concluded that the TiO_2 nanotube arrays with 100 nm diameter not only prompt cell growth and proliferation but also induce osteogenesis expression [15], and as the diameter of TiO_2 nanotube increased from 30 nm to 100 nm, the ALP activity ascends synchronously. Other related researches also indicated that TiO_2 nanotube arrays could significantly promote the adhesion and proliferation of osteoblast cells, as well as accelerated mineral formation in vitro and in vivo [13,18,19]. According to these recent studies, it was obvious that the osteoblast adhesion, proliferation and ALP activity were enhanced significantly by TiO_2 nanotube arrays. However, the range of nanotube diameters in these published studies was limited up to around 100 nm [16], which was attributed to the status quo of fabrication methods [20].

To date, the largest diameter of TiO_2 nanotube could reach 800 nm which was reported by Albu et al., but their TiO_2 nanotubes were not well-organized and seem loosely assembled in a mesoporous matrix rather than the highly ordered nanotube arrays [21]. Yin et al. achieved TiO_2 nanotube arrays with diameter up to about 600 nm [22]. Still, the lack of uniformity made it inappropriate for biological research. As demonstrated in our previous study [23], the highly ordered nanotube arrays with uniform diameters up to 680–750 nm could be obtained via the high voltage anodization process. Thus, the size effects of highly ordered TiO_2 nanotube arrays on osteoblasts could be accessed at larger diameter scales for the first time.

In the present paper, we mainly aimed to explore the in vitro response of osteoblasts towards the large diameter TiO_2 nanotube arrays. A series of TiO_2 nanotube arrays with different diameters (150–470 nm) were fabricated by anodization in the synchronized organic electrolyte. The influence of the large diameter TiO_2 nanotube arrays on the adhesion, proliferation, morphology and osteogenesis expression (ALP

* Corresponding authors.
E-mail addresses: jiahua.ni@sjtu.edu.cn (J. Ni), xnzhang@sjtu.edu.cn (X. Zhang).

http://dx.doi.org/10.1016/j.msec.2015.04.046
0928-4931/

activity) of MC3T3-E1 cells was determined to provide reference for implant optimization. The preparation methods of the large diameter TiO_2 nanotube arrays and the experimental results shown in this paper were expected to provide supports for the influence of TiO_2 nanotube structure on osteoblast behaviors.

2. Materials and methods

2.1. Fabrication of the large diameter TiO_2 nanotube arrays

The annealed cp-Ti sheets with 0.25 mm thickness (99.7% purity, Sigma-Aldrich) were used and cut into 5 mm × 5 mm size. All the experiments in this paper were carried out with this specification. The samples were degreased in acetone with ultrasonication and then chemically etched for 1 min in the mixture of HNO_3 and HF to remove the native oxide film. As demonstrated in reference [23], a two-electrode preparation system with a DC power supply (PSB-2400H, GwINSTEK) was utilized to conduct anodization process. A platinum electrode served as cathode. The electrolyte consisted of 0.5 wt.% NH_4F and 10 vol.% H_2O in ethylene glycol (Sinopharm Chemical Reagent Co., Ltd), which was aged via preliminary usage for 100 h before conducting high voltage anodization. Ti sheets were then anodized at 60 V, 90 V, 120 V, 150 V and 180 V for 3 h, respectively. After anodization, all samples were washed with deionized water followed by a gentle ultrasonication, and then were submerged in the fresh electrolyte for 20 min in order to dissolve the nonuniform porous layer. Half of as-anodized samples were annealed at 500 °C for 3 h in order to transfer the amorphous structure into the anatase phase, which were confirmed by X-ray diffraction (XRD, D/MAX255, Rigaku). All samples were sterilized by autoclaving prior to biological experiments.

2.2. Characterization of surface topography

A field emission scanning electron microscope (FE-SEM, Quanta 250 FEG, FEI) was utilized to observe the surface morphology and to measure the diameters of TiO_2 nanotube arrays anodized at 60 V, 90 V, 120 V, 150 V and 180 V, respectively.

2.3. Measurement of binding strength

A pressure loading test was carried out to identify the binding strength between the substrates and the TiO_2 nanotube arrays. A pull and pressure tester mounted by a 1 mm × 5 mm indenter was utilized during this process. When the nanotube layer started to shatter, the static pressure reading was recorded.

2.4. Contact angle measurement

To assess the wettability of TiO_2 nanotube arrays, an optical contact angle (OCA) measuring device (OCA 20, Dataphysics) was used to measure the water contact angles on the surface of TiO_2 nanotube arrays. Chemically etched Ti sheets were used as a control.

2.5. Surface roughness

In order to quantify the surface roughness of TiO_2 nanotube arrays, an atomic force microscope (AFM, Tapping mode, E-Sweep, Seiko) was used. The average roughness (R_a) was measured for all nanotube structures and Ti foil over a scanning area of 25 μm^2.

2.6. Cell culture

In accordance with the objective of this research, MC3T3-E1 subclone 14 mouse osteoblasts (ATCC CRL-2594, the cell bank of Chinese Academy of Science) were cultured on the surface of TiO_2 nanotube arrays with different diameters to investigate the influence of the large diameter TiO_2 nanotubes on cell behaviors. Each 1 ml original cell suspension was mixed with 6 ml alpha minimum essential medium (α-MEM, Gibco) added with 10 vol.% fetal bovine serum (FBS, Gibco) and 1 vol.% penicillin–streptomycin–neomycin antibiotic mixture (PSN, Gibco). Then the cell suspension was plated in a cell culture flask (430639, Corning Incorporated) and incubated at 37 °C in a humidified atmosphere containing 5 vol.% of CO_2. After 4-day culture, the concentration of MC3T3-E1 cells reached ~3×10^5 cells ml^{-1}. An attenuation process was carried out to set the concentration to 5000 cells ml^{-1}. The cells in the form of cell suspension (500 μl per well) were seeded onto the experimental substrates (TiO_2 nanotubes anodized at 60 V, 90 V, 120 V, 150 V and Ti sheet, respectively), which were placed in the 48-well polystyrene plates directly without rigid fixation and then incubated at 37 °C in a humidified atmosphere of 5 vol.% CO_2 environment for the selected periods of time. All experimental substrates were moved to new 48-well polystyrene plates before cell assays were performed in order to isolate the cells on the substrate of interest from cells on the surrounding polystyrene surfaces.

2.7. Cell counting and viability

In order to count the attached MC3T3-E1 cells on TiO_2 nanotube arrays, the samples were moved into new 48-well polystyrene plates and the cells were detached by trypsin (0.25% Trypsin-EDTA, Gibco) after 6 h, 12 h, 24 h and 48 h of incubation and then counted by a handhold cell counter (Scepter, MILLIPORE).

An MTT (3-(4, 5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide) assay was carried out to estimate the density of viable cells. After 6 h, 12 h, 24 h, 48 h and 7 days of incubation, the substrates with attached cells were washed by the phosphate-buffered saline solution (1 × PBS, Gibco, life technologies) and transferred to the new 48-well culture plates. For each well, 500 μl MTT dye agent (Sigma) was added. After 4 h of incubation in CO_2 incubator, the MTT dye agent was replaced by 500 μl of dimethylsulfoxide (DMSO, Sinopharm Chemical Reagent Co., Ltd) in each well. Then the plates were shaken for 30 min. The absorbance of each well was measured at a wavelength of 570 nm with the subtraction of the 650 nm background by a microplate reader (ENSPIRE 2300, PE) according to the assay instruction.

2.8. Fluorescence observation

In order to observe the cell morphology and elongation, a fluorescein diacetate (FDA, MP Biomedicals) staining was conducted. After 48 h plating on the surface of samples (TiO_2 nanotubes anodized at 60 V, 90 V, 120 V, 150 V and Ti sheet), the cells were washed with PBS. Each 5 mg FDA stock was dissolved in 1 ml acetone and mixed with PBS (10 μl/10 ml). After being incubated in the solution for 1 min and washed again with 1 × PBS, all samples were inverted onto coverslips, visualized and photographed via a fluorescence microscope (Scope.A1, ZEISS) with a green filter. The fluorescence effect was excited by blue laser.

2.9. Cell morphology via SEM

After 48 h of culture, the adhered cells on experimental substrates were washed with 1 × PBS and fixed with 2.5% w/v glutaraldehyde (Sinopharm Chemical Reagent Co., Ltd) in 1 × PBS for 4 h. Then, the samples were washed with 1 × PBS and then dehydrated in a gradient series of ethanol (20, 50, 70, 80, 85, 90, 95, 100 vol.%) for 3 min in each solution. A critical point dryer (EM CPD300, Leica) was utilized to dry the cells on substrates. Finally, the samples were gold-sputter-coated with a sputter coater (EMSCD050, Leica) and were then observed via a field emission scanning electron microscope (FE-SEM, Quanta 250 FEG, FEI).