Applied Surface Science 436 (2018) 653–661

Contents lists available at ScienceDirect

Applied Surface Science

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

Full Length Article

Layered titanates with fibrous nanotopographic features as reservoir for bioactive ions to enhance osteogenesis

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ARTICLE INFO

Article history:
Received 4 August 2017
Received in revised form
27 November 2017
Accepted 5 December 2017
Available online 9 December 2017

Keywords:
Ion exchange
Layered titanate
Bioactive ions
Osteogenesis
Nanotopography

ABSTRACT

In this study, an osteogenic environment was constructed on Ti alloy implants by *in-situ* formation of nanosized fibrous titanate, $Na_2Ti_6O_{13}$, loaded with bioactive ions, i.e. Sr, Mg and Zn, to enhance surface bioactivity. The bioactive ions were loaded by ion exchange with sodium located at inter-layer positions between the TiO_6 slabs, and their release was not associated with the degradation of the structural unit of the titanate. *In-vitro* cell culture experiments using MC3T3-E1 cells proved that both bioactive ions and nanotopographic features are critical in promoting osteogenic differentiation of the cells. It was found that the osteogenic functions of the titanate can be modulated by the type and amount of ions incorporated. This study points out that nanosized fibrous titanate formed on the Ti alloy can be a promising reservoir for bioactive ions. The major advantage of this approach over other alternatives for bioactive ion delivery using degradable bioceramic coatings is its capacity of maintaining the structural integrity of the coating and thus avoiding structural deterioration and potential mechanical failure.

1. Introduction

The construction of an osteogenic microenvironment is critical to enhance osseointegration of bone replacement materials [1,2]. Due to the well-documented importance of bioactive ions in osteogenesis, it has been a highly attractive to utilize them in modern bone biomaterial design. Among bioactive ions, strontium (Sr), magnesium (Mg), and zinc (Zn) have been proved to promote new bone formation and widely used in chemical modification of bone biomaterials [3–10]. Sr, a trace element chemically close to calcium, is known to promote new bone formation by enhancing bone-forming cells activity and meanwhile reducing bone-resorbing cells activity [11]. In our previous work, we found that silicates containing Sr significantly accelerated bone formation at bone fracture sites [12]. One of the most common approaches to endow the implant surface with an ability to release bioactive ions is to coat biodegradable mineral materials composed of the desired ions onto the implant [13–15]. Ions can be released upon the degradation of the mineral materials, however, the structural integrity of the surface coating is inevitably compromised, incurring the possibility of mechanical loss and even delamination of the coating. In addition, to preserve the long-term stability of the implant, the degradation rate of the surface coating is required to perfectly coordinate with the rate of the new bone formation. This is, however, beyond the capabilities of current techniques.

To overcome the side effects caused by the degradation of the surface coating while making the best use of the bioactive ions, we herein propose to use a chemical stable material with an ability to selectively release bioactive ions. For this aim, we selected sodium titanate nanofibers, *in-situ* formed on Ti alloy discs, as a reservoir for bioactive ions. Titanates exhibits excellent chemical and thermal stability, being widely used in water treatment and photocatalysis [16,17]. More recently, Ciofani et al. that barium titanate promoted the osteogenic differentiation of mesenchymal stem cells [18]. They consist of an array of three TiO_6 zig-zag octahedron that share edges to form a chain where sodium ions reside in the inter-layer position between the TiO_6 slabs [19–21]. The alkali metal or alkali earth metal ions in titanates are exchangeable with other cations [20–24], making them a potential ideal reservoir for bioactive ions, such as Sr [16,25]. Due to the chemical stability of the TiO_6 slabs in

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https://doi.org/10.1016/j.apsusc.2017.12.027
0169-4332/

titanates, the release of Sr is not accompanied by the degradation of the titanate. Therefore, titanates have great potential to perform a selective release of bioactive ions while maintaining the structural integrity of the implant surface. Moreover, recent studies proved that engineered nanotopography can facilitate cell adhesion, promote proliferation, initiate intracellular signaling, provide contact guidance and mediate stem cell differentiation [26–29]. Titanates formed by hydrothermal methods normally exhibit nanofiber and nanoflake-shapes [30,31], which makes them particularly appealing in the surface modification of orthopaedic implants, as they can mimic the nanotopography of extracellular matrix (ECM). Based on the above-mentioned merits of titanates, we are interested in exploration of their potential for enhancing osseointegration.

2. Experimental section

2.1. In situ *formation of titanate nanostructure*

Commercially available Ti–6Al–4V discs (Baoji Junhang Metal Material Co., Ltd. Shanxi, China) with a diameter of 15mm and a thickness of 1mm were used. Prior to use in any procedure, both kinds of discs were pre-washed with diluted acid solution containing 20 mL pure water (H_2O), 0.02 mL 48% hydrofluoric acid (HF), and 0.13 mL 48% nitric acid HNO_3. Then, discs were ultrasonically washed with millipore pure water in an ultrasonic bath. To produce titanate nanofibers on the Ti alloys, the pre-washed samples were placed in teflon lined stainless steel autoclave containing sodium hydroxide (NaOH) solution at different concentrations 1 M, 3 M, 5 M and 7.5 M and undergo hydrothermal reactions for 3 h and 6 h. To remove the residual NaOH, the discs were ultrasonically washed in absolute ethanol. Unless otherwise mentioned, the reaction temperature in this study were 60 °C or 180 °C

2.2. Ion-exchange with bioactive ions

Sr, Mg and Zn ions are incorporated into modified Ti alloy by ion exchange with Na ions in the newly formed titanates after hydrothermal alkaline treatment. Briefly, 250 mM metal chloride solutions ($SrCl_2$, $ZnCl_2$ and $MgCl_2$) with a pH value of 5, 7, 9 and 11 were prepared. Then, the alkaline treated Ti alloy discs were immersed in a certain metal chloride solution for 3 h and 6 h, followed by rinsing with mili-Q water. Atomic ratios of Sr/Ti, Mg/Ti and Zn/Ti were determined using energy-dispersive X-ray spectroscopy (EDS) attached to a SEM microscope (ZEISS SUPRA® 55).

For competitive ion exchange experiments, any two of the above mentioned metal chloride solutions with a pH value of 5.0 were mixed. For example, 250 mM $SrCl_2$ and 250 mM $MgCl_2$ were mixed for test of competitive ion exchange with Na ions between Sr and Mg ions. The samples were immersed in the mixed salt solution for six hours and then rinsed with milli-Q water.

2.3. Bioactive ions release

The release behavior of bioactive ions incorporated in the titanate layer after ion exchange was studied in the media of physiological saline NaCl solution and cell culture medium at 37°C within 1, 2 and 3 days. Briefly, the Ti alloy discs were placed in 24-well cell culture plates and 1mL cell culture medium or physiological saline solution were added into each wells. All the samples were incubated for 1, 2 and 3 days at 37 ºC, in a 100% humid environment. At each time point, the solution was collected, and the well was refilled with an equivalent volume of fresh saline solution or cell medium. The ion concentration in the collected media was measured by inductively coupled plasma mass spectrometry (PE ICP-OES Optima 7000DV).

2.4. Cell culture and seeding

Preosteoblasts (MC3T3-E1) cells were used to evaluate the cytocompatibility and bioactivity of the Ti-6Al-4V alloy after surface modification. Briefly, cells were cultured using α-MEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C, 5% CO_2 in a humidified environment, following a procedure reported in our previous papers. The specimens were first sterilized by autoclave sterilization at 121 °C for 20 min before cell seeding, and then placed in 24-well tissue culture plates. 1mL of cell suspension with a density of 2×10^4 cells/mL was seeded onto the specimens.

2.5. Cellular attachment and adhesion

To investigate the effect of the surface treatment on the cellular attachment and adhesion, cells were seeded at a density of 4.0×10^4 cells/mL. After 6h and 24h incubation, cells were fixed with 2.5% glutaraldehyde solution and dehydrated in a series of gradient concentration of ethanol (50, 70, 80, 90, 95 and 100, v/v%). Finally, the specimens were dried by hexamethyldisilazane (HMDS) and sputter-coated with platinum before observation using scanning electron microscopy (SEM, S-4800, Hitachi High Technologies).

To further observe the cell adhesion under the influence of the surface treatment, cytoskeleton protein (F-actin), nucleus and vinculin were stained and observed using confocal laser scanning microscope (CLSM, preformed on Zeiss LSM 510). Briefly, after culturing for 6 h and 24 h, cells were fixed in a 4% paraformaldehyde solution, and permeabilized with Triton-X100 (Sigma, USA) for 4 min at room temperature. Then, cells were incubated in dilute primary antibody (anti-vinculin) solution overnight at 4 ºC, followed by three times washes for 5 min each with phosphate buffered saline (PBS), followed by 45 min further incubation with secondary antibody fluorescein isothiocyanate (FITC)-conjugated and tetramethylrhodamine (TRITC)-conjugated Phalloidin at room temperature. After three times washes with PBS, the cells nucleus was stained using 4′,6-diamidino-2-phenylindole (DAPI) for 5 min at room temperature.

2.6. ALP activity

Alkaline phosphatase (ALP), an indicator of early differentiation of osteoblast-like cells, was measured after the cells were cultured on the treated alloy surface. After 7 and 14 days, the cell culture medium was removed, and 200 μL of specific buffer (20 μL PMSF, 1.98 mL Triton X-100) was added to each well at room temperature and incubated for 1 h to obtain cell lysates. Then, 50 μL of the cell lysates from each sample was added to 96-well plates. 50 μL p-nitrophenyl phosphate substrate solution (Sangon, Shanghai, China), containing 0.1 mol/L glycine and 1 mmol/L $MgCl_2 \cdot 6H_2O$, was added and incubated for 2 h at 37 °C. The reaction was quenched by adding 100 μL NaOH (0.1 mol/L) and the absorbance of the solution was quantified at 405 nm using a microplate reader. The total protein content in each cell lysates was determined using a BCA assay kit. ALP levels were normalized to the total protein content, and all experiments were performed in quadruplicate.

2.7. Quantitative real-time reverse-transcriptase polymerase chain reaction

Cells were seeded on surface treated Ti alloy specimens ($\phi = 21$ mm) placed in 12-well cell culture plate, at an initial density of 1×10^5 cells/well. After 24h of incubation, culture medium was exchanged for osteogenic differentiation media consisting of 10 mM b-glycerophosphate, 50 mg/mL ascorbic acid and 0.1 mM dex-