Materials Science & Engineering C 94 (2019) 1–10

Contents lists available at ScienceDirect

Materials Science & Engineering C

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

Nanostructured titanium surfaces fabricated by hydrothermal method: Influence of alkali conditions on the osteogenic performance of implants

Yi-Zhou Huang[a,b], Shu-Kun He[a,b], Zhi-Jun Guo[c], Jin-Kui Pi[a], Li Deng[a], Li Dong[a], Yi Zhang[a], Bo Su[d], Lin-Cui Da[a], Li Zhang[c], Zhou Xiang[b], Wei Ding[a], Mei Gong[a,*], Hui-Qi Xie[a,*]

[a] Laboratory of Stem Cell and Tissue Engineering, State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu 610041, PR China
[b] Department of Orthopedics, West China Hospital, Sichuan University, Chengdu 610041, PR China
[c] The Research Center for Nano Biomaterials, Analytical & Testing Center, Sichuan University, Chengdu 610064, PR China
[d] Core Facility, West China Hospital, Sichuan University, Chengdu 610041, PR China

ARTICLE INFO

Keywords:
Alkali concentrations
Bone regeneration
Hydrothermal method
Nanostructured surfaces
Osteointegration
Titanium

ABSTRACT

Hydrothermal method is an easy-to-use approach for creating nanostructured surfaces on titanium (Ti). However, whether the alkali conditions of this method influence the osteogenic potential of the modified surfaces remains unknown. In this study, we fabricated nanostructured surfaces, termed the Ti-1, Ti-5, and Ti-10 groups, by using the hydrothermal method in 1 M, 5 M, and 10 M NaOH aqueous solutions, respectively. An untreated Ti surface served as a control. The osteogenic performance of modified surfaces was systemically investigated, including the proliferation and osteogenic differentiation of human osteoblast-like MG63 cells *in vitro* and the osteointegration of implants in a rabbit femoral condyle defect model. After hydrothermal treatment, the hydrophilicity of modified surfaces was greatly enhanced. The Ti-1 group showed a nanowire-like topography, while the Ti-5 and Ti-10 groups exhibited a nanopetal-like topography with different pore sizes. Compared with the untreated Ti surface, the modified surfaces showed good cytocompatibility and enhanced the osteogenic differentiation of MG-63 cells. Compared with the other modified surfaces, the Ti-5 group was the most favourable for the osteogenic differentiation of cells, showing higher levels of alkaline phosphatase activity, osteogenic gene expression, mineralization and osteoprotegerin secretion. Twelve weeks after implantation at the bone defects, the Ti-5 group showed superior peri-implant bone regeneration and higher peak push-out force than the other groups. Overall, this study revealed the crucial role of alkali conditions of hydrothermal method in modulating the material characteristics of modified surfaces and their osteogenic performance *in vitro* and *in vivo*, highlighting the need for optimizing the processing conditions of hydrothermal method for enhanced osteointegration.

1. Introduction

Titanium (Ti) and its alloys are among the most commonly used materials for dental and orthopaedic implants, based mainly on their good biocompatibility, high corrosion resistance and excellent mechanical properties [1,2]. Nevertheless, they are bioinert materials, and the lack of bioactivity often leads to fibrous capsule formation at the bone-implant interface [3,4], resulting in post-implantation micromotions and, ultimately, a short lifespan or even failure of the implants. In view of these shortcomings, much effort has been expended on improving the bioactivity and osteoinductivity of Ti-based bone implants, with particular focus on developing easy and practicable strategies that facilitate new bone formation at the bone-implant interface.

Surface topography is a crucial factor affecting the osteointegration of bone implants. Microtopography regulates the functions of mesenchymal stem cells (MSCs) [5–7], osteoclasts [8,9], and osteoblasts [8,10–12]. It is well-known that microrough surfaces are good for rapid and improved osteointegration [13–15]. Similarly, nanotopography profoundly influences the responses of bone-related cells, such as cell mobility, proliferation and differentiation [16–20]. Based on this, diverse surface modification strategies, *e.g.* electrochemical deposition [21], anodization [22] and alkali-heat treatment [23], have been utilized to produce specific nanotopography for enhanced osteointegration.

Besides topographic cues, surface chemical properties modulate the osteogenic performance of bone implants, since they dramatically

* Corresponding authors at: No.1 Ke-Yuan-Si-Lu, Gao-Peng-Da-Dao, Chengdu, Sichuan 610041, PR China.
E-mail addresses: maggie-mei1983@163.com (M. Gong), xiehuiqi@scu.edu.cn (H.-Q. Xie).

https://doi.org/10.1016/j.msec.2018.08.069
Received 9 December 2017; Received in revised form 17 August 2018; Accepted 31 August 2018
Available online 03 September 2018
0928-4931/

influence the cell-implant interactions [24–26] and peri-implant bone regeneration [27,28]. According to previous reports, alkali treatment of Ti implants can produce bioactive surfaces that induce bone-like apatite formation when immersed in simulated body fluid [29–31]. Likewise, after an alkaline-and-heat treatment, a bioactive amorphous sodium titanate layer can be introduced on Ti surfaces [32,33], facilitating the osteointegration of implants [34,35].

We recently employed a facile and cost-effective approach, *i.e.*, the hydrothermal method, to obtain nanostructured titanate layers on Ti surfaces *via* a combination of relatively high pressure and temperature [36]. The composition of nanostructured titanate layers is $H_2Ti_2O_5{\cdot}H_2O$ and $Na_2Ti_2O_5{\cdot}H_2O$. By changing the alkali conditions, this method produced surfaces with diverse topographies. The modified surfaces induced bone-like apatite formation in simulated body fluid. When cultured with rat MSCs, the nanopetal-like structures showed an acceptable cytocompatibility and enhanced the osteogenic gene expression of cells *in vitro* [36]. However, the osteogenic performance of this type of nanostructure, especially its *in vivo* osteointegration, remains largely unknown.

By changing the concentration of NaOH, another type of nanostructure, *i.e.*, the nanowires structure, can be obtained *via* the hydrothermal method, but its cytocompatibility and osteogenic performance have not yet been determined. From a clinical point of view, although both the nanowires and the nanopetal-like structures can be produced by a relatively simple approach through changing the alkali conditions, an important question needs to be answered: Which type of nanostructure is more suitable for future clinical applications in bone repair? In other words, do the alkali conditions of hydrothermal method influence the osteogenic performance of implants?

To address these questions, the present work was undertaken through a series of evaluations to determine the osteogenic performance of the aforementioned surfaces *in vitro* and *in vivo*, especially their osteointegration and biomechanical properties after implantation at bone defects. This work will provide useful guidance for choosing the proper processing condition in the hydrothermal method to achieve a good clinical outcome with nanostructured Ti implants.

2. Materials and methods

2.1. Sample preparation and characterization

2.1.1. Sample preparation

Samples were prepared by the hydrothermal method as in our previous work [36]. Briefly, commercially pure Ti substrates (Ti > 99.5%; Baoji Titanium Industry Co. Ltd., Baoji, China) were cut into rectangular plates for *in vitro* study or rods (Φ 4 mm × 9 mm) for *in vivo* study. To remove the oxide layer on the surface of Ti substrates, samples were washed with a mixture solution of HF: HNO_3: H_2O_2 (V/V/V = 1:3:5) for 5 min. Next, the samples were ultrasonically cleaned sequentially in acetone, 2-propanol, alcohol and deionized water for 30 min, respectively, and dried in a vacuum freeze dryer. Afterwards, the samples were divided into four groups, namely the Ti, Ti-1, Ti-5, and Ti-10 groups. No additional treatment was applied to the Ti group, which served as the control. For the Ti-1, Ti-5, and Ti-10 groups, samples were immersed in 1 M, 5 M, and 10 M NaOH aqueous solutions, respectively, and treated in a Teflon-lined autoclave with a pressure of 0.2 MPa at 110 °C for 5 h. After the hydrothermal treatment, samples were thoroughly washed with deionized water, dried in air at room temperature (RT) for 24 h, and used in the following experiments.

2.1.2. Field emission scanning electronic microscopy

Surface topography was examined by a field emission scanning electronic microscopy (FESEM) (Nova NanoSEM 450, FEI, USA) with an accelerating voltage of 15 kV, a working distance of 4.9 mm and a magnification of 120,000. Representative images were chosen to qualitatively assess the size of pores among the nanostructures by using the National Institute of Health's (NIH) Image J software (NIH, Rockville, MD, USA). Four different fields of each image were measured, with at least 100 pores evaluated in each group.

2.1.3. Contact angle measurement

Surface wettability was detected by a contact angle measurement (JY-82A; Dingsheng Testing Instrument Co. Ltd., Chengde, China). A droplet of deionized water was deposited on the surface of each sample. Images were taken 15 s after stabilization of the droplet. At least eight samples were analysed for each group.

2.2. In vitro studies

2.2.1. Cell viability and proliferation

The samples were placed in 24-well plates. MG63 cells were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were seeded at a density of 1×10^4 cells/cm^2 and cultured in the growth medium containing Dulbecco's modified Eagle's medium-high glucose (DMEM-HG) (Gibco Laboratories, Gaithersburg, MD, USA), 10% foetal bovine serum (FBS) (Hyclone, Logan, UT, USA), and 1% penicillin/streptomycin (Gibco Laboratories, Gaithersburg, MD, USA).

A live/dead staining kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) was used to determine cell viability. Briefly, samples were stained using the kit for 15 min in the dark at 37 °C. Afterwards, the samples were washed with phosphate buffer saline twice and viewed under a confocal laser scanning microscopy (Nikon, NY, USA).

A cell counting kit (CCK-8, Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to monitor cell proliferation. Briefly, 30 μL of CCK-8 solution and 300 μL of DMEM-HG medium were added to each sample and incubated at 37 °C for 2 h. Then, 100 μL of the supernatant were transferred to a 96-well plate, and the absorbance at 450 nm was immediately read using a micro-plate reader (Molecular Devices LLC, Sunnyvale, CA, USA).

2.2.2. Alkaline phosphatase (ALP) activity

MG63 cells were seeded at a density of 5×10^3 cells/cm^2 and cultured in the growth medium without any osteogenic inductive factors. After 7 days of culture, the ALP activity of cells was evaluated using an ALP activity kit (Nanjing Jiancheng Bio-engineering Institute, Nanjing, China) according to the manufacturer's instructions. The ALP activity of cells was normalized to the total protein quantified using a BCA protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

2.2.3. Real-time polymerase chain reaction (RT-PCR)

MG63 cells were seeded at a density of 5×10^3 cells/cm^2 and cultured in the growth medium without any osteogenic inductive factors. After culture for 1, 3 and 7 days respectively, total RNA of the cells was extracted using an RNAiso Plus reagent (Takara Biotechnology, Tokyo, Japan) and reverse-transcribed into complementary DNA (cDNA), using a PrimeScript RT reagent Kit (Takara Biotechnology, Shiga, Japan). Gene expression was quantified using a SYBR Premix Ex Taq II kit (Takara Biotechnology, Otsu, Japan) in an IQ5 real-time system (Bio-Rad, Hercules, CA, USA). Primers of target genes, including *ALP*, *type 1 collagen* (*Col 1*), *runt-related transcription factor 2* (*Runx2*) and *osteoprotegerin* (*OPG*), are listed in Table 1. The relative expression of target genes was normalized to the expression of the housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (*GADPH*). Data were analysed by the $2^{-\triangle\triangle Ct}$ method. Results were expressed relative to the gene expression of the Ti group.

2.2.4. Mineralization of the extracellular matrix

MG63 cells were seeded at a density of 5×10^3 cells/cm^2 and cultured in the osteogenic medium that contained the growth medium, 50 mg/L L-ascorbic acid-2-phosphate (Sigma-Aldrich, St. Louis, MO, USA), 10^{-4} mM dexamethasone (Sigma-Aldrich, St. Louis, MO, USA)