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ARTICLE

In vitro Apatite Formation, Protein Adsorption and Initial Osteoblast Responses on Titanium Surface Enriched with Magnesium

Xu Lingli, Shi Xingling, Ouyang Chun, Liu Wen

Jiangsu University of Science and Technology, Zhenjiang 212003, China

Abstract: A feasible treatment to modify titanium with Mg was carried out. Briefly, pure titanium was treated in condensed sodium hydroxide solution first and then transferred to diluted magnesium chloride solution to conduct ion exchange. After that, heat treatment was conducted and a nano-sized network containing magnesium titanate was obtained. Surface morphology, roughness, and chemical composition were characterized. *In vitro* apatite inducing ability was evaluated in simulated body fluid (SBF) and bovine serum albumin (BSA) was used as model to study protein adsorption. MC3T3-E1 cells were cultured and initial cell attachment, morphology, proliferation were evaluated. Results show that compared with sodium (Na) modified surface, Mg immobilization accelerates apatite formation and promotes protein adsorption significantly. Besides, cell attachment is improved and cell spreading is enhanced on Mg-containing samples compared with Na containing samples. Increased early cellular attachment results in subsequent increase of number of proliferated cells on the Mg-containing surface.

Key words: titanium; osteoconductivity; magnesium; apatite; protein; osteoblast

Pure titanium and its alloys are widely used as bone substitute in dentistry and orthopedics due to their good corrosion resistance and proper mechanical strength matching with bone[1, 2]. However, as titanium is bioinert material it cannot bond directly to living bone and fibrous capsule always formed isolating the implant from surrounding bone [3]. Initial osseointegration is considered to be crucial for long-term implantation success, and thus surface modification of medical titanium and its alloys for rapid and firm osseointegration has been research interest since they were used as load-bearing bone substitute.

Protein adsorption is the most immediate event after implantation and is critical for the biocompatibility of all implant materials. Cell adhesion and subsequent proliferation, differentiation are mediated by interactions between cell integrins and adsorbed proteins and thus the amount and type of protein absorbed onto implants surface consequently affect the behaviors of osteoblasts[4, 5]. Magnesium (Mg) is the fourth most abundant mineral in human body and Mg ions are known to enhance adhesion of osteoblast via ligand binding of the integrin receptors[6]. In order to improve protein adsorption, titanium implants surface are modified with Mg through various techniques, including electrochemical oxidation, hydrothermal treatment and ion implantation[7-12].

For implants with complex shapes, hydrothermal treatment and electrochemical oxidation are preferred since uniform bioactive oxidation layer with certain cations can be formed all over the titanium surface. The amount of interesting element immobilized onto titanium was limited by hydrothermal method[13,14]. Increasing the pH of hydrothermal medium will benefit incorporation of cations; however, it also causes the precipitation of hydroxide of alkaline element, such $Mg(OH)_2$, which will depress cell responses[15,16]. Besides, hydrothermal treatment and electrochemical oxidation need complex devices to supply de-

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Corresponding author: Shi Xingling, Ph. D., School of Material Science and Engineering, Jiangsu University of Science and Technology, Zhenjiang 212003, P. R. China, Tel: 0086-511-84401184, E-mail: shixingling1985@hotmail.com

fined electric filed or to guarantee reaction at high pressure under temperature as high as 200 °C.

It was concluded that the formation of titania gel would benefit the adsorption divalent cations that show much stronger affinity towards titania compared with univalent ones[17]. Thus, it is possible to replace the Na combined into titanium surface during alkaline treatment with a large amount of other bioactive elements such as zinc (Zn), calcium (Ca) and Mg. In the present work we prepared titanium specimens enriching in Mg by an alkaline-immersion-heat treatment method and the effects of Mg cations on apatite formation, protein adsorption and cell responses were evaluated in comparison with Na enriched specimens.

1 Experiment

Commercially pure titanium disks (Kobe Steel Ltd, Japan), *Φ*15 mm ×1.0 mm, were used in the experiments. Samples were wet-abraded with 400-grit SiC abrasive paper and then ultrasonically washed in acetone and distilled water for 30 min. After dried in hot air, Ti samples were immersed in 5.0 mol/L NaOH solution at 60 °C for 24 h and then were washed by distilled water thoroughly. Alkaline-treated samples were either dried in hot air or immersed into 0.1 mol/L $MgCl_2$ at 60 °C for another 24 h. Finally, further immersed samples were rinsed by distilled water and dried in hot air. After chemical treatment, samples were placed into a muffle furnace and subjected to a heat treatment at 600 °C in air for 1 h. The temperature rising speed was 5°C/min. Samples were coded with regard to the cations, namely, NaTi for NaOH-heat treated and MgTi for NaOH-$MgCl_2$-heat treated. Untreated titanium discs were coded as UnTi.

1.1 Surface characterizations

Surface morphologies of the samples were observed with scanning electronic microscope (SEM, S-3000, Hitachi Co., Japan). Surface roughnesses of samples were analyzed by color 3D laser scanning microscope (VK9710, Keyence Co., Japan).The surface chemical compositions of samples were detected by X-ray photoelectron spectroscopy (XPS, K-alpha, Thermo Fisher Scientific, UK). Crystalline structures in the top layer of samples were investigated by grazing incidence X-ray diffraction (GIXRD, D8 Advance, Bruker AXS Inc., USA). The diffraction data were recorded at an X-ray incidence angle of 4°.

1.2 *In vitro* apatite induction

Samples were put into individual PE bottles containing 35 mL simulated body fluid (SBF) and were kept in an incubator set at 36.5 °C as proposed by Kokubo et al[18]. After 2 d of incubation, samples were taken out, rinsed gently with distilled water and then dried in hot air at 60°C. The surfaces of samples after immersed in SBF were characterized by SEM.

1.3 Protein adsorption

Bovine serum albumin, fraction V (BSA, Pierce Biotechnology Inc., Rockford, IL) was used as a model protein and Bradford method was employed. 200 μl of protein solution (1 mg of protein in per mL of saline) was spread over Ti disks lying on the bottom of 12-well cell culture plate using a pipette. The adsorption was then conducted in a sterile humidified incubator at 37°C for 3h. After incubation, 100 μL of protein solution from top of each sample was mixed with 5.0 mL 5-times diluted assay solution. 200 μL of the mixture from each sample was pipetted into a well of 48-well plate and 3 replicates for each sample were used. The samples were incubated at room temperature for 5 min and absorbance was read at 595 nm using an auto microplate reader (Infinite M200, Tecan, Austria). Calibration curve was prepared by measuring diluted series of standard solution.

1.4 Cell culture

MC3T3-E1 cells, a mouse calvaria-derived osteoblast-like cell line, were cultured in L-glutamine containing alpha-Minimum Essential Media (α-MEM, GIBCO/Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA), 1% penicillin (10000 units) and streptomycin (10 mg/mL). The cells were cultured under 100% humidity and 5% CO_2 at 37°C. The medium was changed every other day and cells were passaged using 0.05% trypsin/0.02% EDTA (Invitrogen, Carlsbad, CA, USA) prior to confluenced.

1.5 Initial cell attachment and proliferation

Cells were seeded onto Ti samples in 24-well plate at an initial density of 4×10^4 cells/well. After cultured for 3 h and 24 h cells were washed with phosphate buffer saline (PBS) and trypsinized. The attached cells were then counted by hemocytometer.

After 3 h of culture, cells attached to the disks were fixed with 3% glutaraldehyde, post fixed with 1% OsO_4 solution and then dehydrated using a series of ethanol solution with graded concentration. Subsequently, they were dried with the *t*-butyl alcohol freeze-drying method and sputtered with Au-Pt alloy coating for SEM observation.

For proliferation, cells were seeded on disks in 24-well plates at 1×10^4 cells/well and Alamar Blue assay (Biosource International, Camarillo, CA) was performed after cultured for 1, 3, 5 and 7 d. At each time point, the medium was replace by 1.0 mL culture medium with 10% Alamar Blue reagent and the cells were further incubated for 3 h. 100 μL (3 replicates for each sample) of the medium from each well was transferred to a 96-well plate and the fluorescence (λ_{ex} = 560 nm, λ_{em} = 590 nm) was read. The remaining Alamar Blue in well-plate was washed away with α-MEM and fresh culture medium was added for further incubation.

1.6 Statistical analysis