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Jiang, N., Guo, Z., Sun, D., Ay, B., Li, Y., Yang, Y., Tan, P., Zhang, L., & Zhu, S. (2019). Exploring the mechanism behind improved osteointegration of phosphorylated titanium implants with hierarchically structured topography. *COLLOIDS AND SURFACES B-BIOINTERFACES*, 184, [110520].
<https://doi.org/10.1016/j.colsurfb.2019.110520>

Published in:
COLLOIDS AND SURFACES B-BIOINTERFACES

Document Version:
Peer reviewed version

Queen's University Belfast - Research Portal:
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Exploring the Mechanism behind Improved Osteointegration of Phosphorylated Titanium Implants with Hierarchically Structured Topography

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Abstract

Titanium (Ti) and its alloys have been frequently used in dental and orthopedic implants, but the undesired oxide layer easily formed on the surface tends to be the cause of implant failure for Ti-based implants. To address this problem, we herein prepared a phosphorylated Ti coating (TiP-Ti) with a micro/nano hierarchically structured topography on commercially pure Ti implants by a hydrothermal method to improve its osteointegration capacity. The surface morphology, chemical composition, and biological activity were characterized by scanning electron microscopy (SEM), transmission electron microscopy (TEM), X-ray diffraction (XRD), X-ray photoelectron spectroscopy (XPS), atomic force microscopy (AFM), contact-angle measurement, and protein adsorption assay. Osteointegration of TiP-Ti implants in rat tibia was investigated by biomechanical testing, micro-CT and histological analyses. We further explored the proposed mechanism which improves osteointegration of TiP-Ti implants by proliferation, adhesion, and differentiation assays of rat bone marrow mesenchymal stem cells (BMSCs). Our results demonstrated that the improved osteointegration mainly benefited from the better spread and adhesion of BMSCs on the micro/nano hierarchically structured TiP-Ti surfaces compared to hydroxyapatite coated Ti (HA-Ti), the positive control, and untreated Ti (untreated-Ti), the negative control. In conclusion, TiP-Ti surface is a promising candidate implant surface design to accelerate the osteointegration of Ti-based implants in biomedical applications.

Key words: osteointegration; surface modification; hierarchical structure; mechanism; titanium implant

1. Introduction

Titanium (Ti) and its alloys have been frequently used as biomaterials in dentistry and orthopedics. They show superior properties in mechanical strength, toughness, corrosion resistance, and biocompatibility; however, the presence of the stable oxide layer on their surface makes them bio-inert in physiological environments [1–4]. Due to the bio-inertia, traditional pure Ti lacks initial osseointegration, leading to the increased aseptic loosening and subsequently early failure of the implants [5–7]. To improve the bioactivity and accelerate osseointegration of Ti implants, many surface modifications, such as apatite coating, surface protein-grafting, and surface roughening, have been studied [8–12]. However, the issues of peeling of the coating, foreign body reaction, and release of metallic cations are still concerned since these complication are known to reduce the long-term stability of Ti-based implants *in vivo* [13–15].

Natural bone tissue has a micro/nano scaled hierarchical structure and its mineral phases are mainly composed of calcium (Ca) and phosphorus (P) elements [16–19]. In the previous studies, micro/nano-scaled hierarchical coatings containing Ca and P have been proven to significantly improve cell viability, adhesion, and differentiation, which subsequently lead to enhanced osseointegration and improved stability of the implants [20–25]. More recently, our group reported that the implants with micro/nano-scaled hierarchical hybrid morphology containing Ti phosphates and Ti oxides demonstrated a remarkable performance both *in vitro* and *in vivo* compared to untreated commercially pure titanium [26]. However, the attempts to have an in-depth understanding of how the hierarchical topography influence the interactions between cells and material surfaces are still in progress, and the mechanisms of surface modifications in promoting osteointegration is not yet clear.

In the past decade, Ti implants with hydroxyapatite (HA, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) surface coating, such as Zimmer TSV-HA (Zimmer Dental, Carlsbad, CA, USA) and Osstem TS III-HA (Osstem Implant Co. Busan, Korea), have been successfully commercialized to be used as clinical oral implants amongst various treatments. Indeed, many pyro processing methods of forming HA and other calcium phosphate coatings on metallic

substrates have been reported (e.g., plasma spraying [27], sol-gel method [28], electron beam sputtering method, and ion beam sputtering method [29]. However, plasma spraying remains the most commonly used technique for HA coating on a Ti or Ti alloy substrate commercially in the fabrication of artificial joint replacements [30] and in endosseous dental implants [27], because of its beneficial effects on rapid bone ingrowth and increasing lifespan of the orthopedic prosthesis [31,32]. However, numerous reports [33,34] indicated instability of the plasma-sprayed HA which can easily decompose into other phases, such as tricalcium phosphate, calcium oxide, tetracalcium phosphate and amorphous calcium phosphate. Some of these compounds are undesirable due to fast dissolution *in vivo*. Additionally, severe cracking of the plasma-sprayed layer (an inherent feature of plasma-sprayed ceramics) frequently leads to accelerated implant failure [35]. Recently, HA coatings produced in our group deploying sol-gel approach have demonstrated significantly enhanced structural integrity that helps to overcome the inherent weakness of plasma sprayed HA [36] .

We therefore used sol-gel HA coated Ti (HA-Ti) following our established technique as the standard surface modification and the *in vitro* and *in vivo* performance of HA-Ti compared with the micro/nano-scaled hierarchical hybrid Ti surface containing Ti phosphates and Ti oxides [26]. The purposes of this study include: (1) to prepare a micro/nano-scaled phosphorylated Ti surface (TiP-Ti) and determine its topographical and chemical features; (2) to investigate the osteointegration capacity of micro/nano-structured TiP-Ti on osseointegration by *in vivo* tests, biomechanical testing, micro-CT assay, and histological analysis, in comparison with HA-Ti and pure titanium (untreated-Ti); (3) to compare the bioactivity of TiP-Ti surfaces with HA-Ti and untreated-Ti implants and explore the mechanism by which TiP-Ti surfaces improve osseointegration through *in vitro* tests on cell adhesion, proliferation, and differentiation.

2. Materials and Methods

2.1 Sample Preparation and Characterization

2.1.1 Sample Preparation

Both rod-shaped (diameter: 1 mm; length: 12 mm, for *in vivo* experiment) and disc-shaped samples (diameter: 10 mm; thickness: 1 mm, for *in vitro* experiment) used in this study were made of pure Ti (manufactured at the Institute of Metal Research, Chinese Academy of Sciences, Liaoning, China) with three different surface modifications. All samples were polished to a mirror finish using 800, 1200, 2000 grit silicon carbide sandpapers, respectively and this resulted in the “Untreated-Ti” group. An untreated-Ti group was further modified by sol-gel dipping-drying-firing process following our previously established protocol [26] and formed the “HA-Ti” group. Briefly, analytical grade $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (Sigma-Aldrich) and P_2O_5 (Sigma-Aldrich) were dissolved in absolute alcohol in two separate beakers and serve as Ca and P precursor. The two precursors were then mixed by a Ca/P molar ratio of 10/6 and refluxed for 24 h to form dipping sols. Thereafter, a batch of untreated Ti implants were immersed into the dipping sols and withdrawn at a speed of 8 cm/min. After 15 min drying at 150 °C and 15 min firing at 700 °C, a single layer of 10% HA was obtained. Such dipping-drying-firing process was repeated twice to obtain a desired coating thickness. A second batch of untreated-Ti group was modified by a hydrothermal reaction under 0.15 MPa pressure in a mixture of 4.5 wt% aqueous phosphoric acid and 9wt% hydrogen peroxide at 120 °C for 24 h and named as “TiP-Ti” group.

2.1.2 Surface Topography

The surface morphology of samples was observed using both a transmission (Tecnai G2 F20, FEI, Netherlands) and a scanning electron microscope (Inspect F; FEI, Hillsboro, OR, USA), and the elements on surface were detected by an energy-dispersive X-ray spectrometer (EDS, QX200, Bruker, USA) attached on the SEM with an acceleration voltage of 20 kV. The chemical states of surface elements were determined by an X-ray photoelectron spectrometer (XPS, AXIS Ultra DLD, Kratos, UK) outfitted with a monochromatic Al K α radiation ($h\nu=1486.6$ eV). Surface topography of samples was evaluated by an atomic force microscope (AFM, Shimadzu

Corporation, Japan) on three samples in each group (five random positions per sample). Surface roughness values were assessed by the Nanoscope Multimode software.

2.1.3 Phase and Chemical Composition

The elemental compositions and crystallographic structures of the samples were investigated with a powder diffractometer (X'Pert Pro-MPD, Philips Analytical, Netherlands) with a scanning speed of 0.75°/min and an angle range of 10–80° for phase composition analysis.

2.1.4 Surface Energy

The surface energy was assessed based on surface wettability while employing a contact angle assay using an automated goniometer/tensiometer (Model 290, RaméHart instrument co., USA) with three samples in each group (five random positions per sample).

2.1.5 Protein Adsorption Assay

Disc-shaped Ti samples were placed into 24-well plates, rinsed with PBS, and incubated at 37°C for 1 hour with PBS containing 10% FITC-conjugated albumin from Bovine Serum (Gibco, Thermo Fisher Scientific, USA). Samples were then rinsed with deionized water to remove unattached proteins. Adsorbed proteins were visualized by both SEM and confocal laser scanning microscope (CLSM, LSM700, Carl Zeiss, Germany). Surface coverage of adsorbed proteins was calculated by the CLSM images using Image J software (NIH, USA).

2.2 Animal Study

2.2.1 Animal Model and Surgery

Sixty female Sprague Dawley rats (3-month-old, 230-270gr) were divided into 3 groups, and a total of 120 rod-shaped implants (diameter: 1 mm; length: 12 mm) (n=40 samples per group: Untreated-Ti, HA-Ti, and TiP-Ti) were placed bilaterally in the distal metaphyses of rat tibia as described previously [37]. Briefly, after anesthesia was applied, a 1-mm hole was made on the condylar notch with a rotary drill and the samples were inserted into the medullary canal of the tibial metaphyses.

2.2.2 Biomechanical Testing

Biomechanical testing of the specimens (10 tibias/group) was performed to assess maximum push-out force (N) and ultimate shear strength (N/mm²) using a universal material testing system (Instron 5566, USA) as previously described [38]. The application speed was set as 1 mm/min.

2.2.3 Micro-CT Evaluation

Specimens (20 tibias/group) were investigated by a μ -CT scanner system (Scanco Medical μ -CT 50, Switzerland). The volume of interest (VOI) was defined as 2 mm below the proximal growth plate to a distal 100 slices with a ring radius of 200 μ m from the implant surface. 3D reconstructed images were then generated, and the values of bone volume per total volume (BV/TV), percentage of osteointegration (%OI), mean trabecular number (Tb.N), and mean trabecular separation (Tb.Sp) were calculated within the VOI. %OI was calculated.

2.2.4 Histological Analysis

The region-of-interest of samples (10 tibias/group) was defined as approximately 2 mm below the proximal growth plate, with a ring radius of 200 μ m from the implant surface. Bone area ratio (BA, defined as the percentage of mature bone to the whole tissue region) and bone implant contact (BC, defined as the percentage of mineralized bone in direct contact with the implant interface) were calculated.

2.3 *In vitro* Cell Behaviors

2.3.1 Cell Proliferation

Cell proliferation capacity of passaged BMSCs on the disc-shaped samples were determined using a 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (MTT, M-2128, Sigma-Aldrich, USA) following the culture of BMSCs for 1, 3, 5, 7, and 9 days in the growth media (α -MEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA). The optical density was measured using a spectrophotometer (Anthos 2010, Biochrom, UK) at an excitation wavelength of 490 nm. Furthermore, another batch of the BMSCs cultured for 3 days were collected for

flow cytometry (FCM) to investigate the cell cycle with a flow cytometer (Accuri C6, BD Biosciences, USA). Flow cytometric analysis of cell cycle was performed in compliance with the protocol recommended by the manufacturer (BD Biosciences).

2.3.2 Cell Morphology

Changes in cell morphology on the disc-shaped Ti samples were observed by SEM, AFM, and CLSM separately. Briefly, BMSCs were cultured on disc-shaped Ti samples in 24-well plates for 1 and 6h. They were then fixed with 4% paraformaldehyde for 30 min, rinsed with PBS twice, dehydrated with graded ethanol series (from 20% to 100%), and gold sputtered for SEM to visualize the distribution of the cells on the Ti samples. Another batch of fixed and dehydrated cells were investigated with AFM (under the contact mode, Nanoscope MultiMode, Veeco Instrument, USA) to identify the pseudopodia of the cells. A second batch of cells cultured on the Ti samples were fixed in paraformaldehyde, stained with DAPI (Abcam, USA) and rhodamine phalloidine (Millipore, USA) to be imaged with CLSM. CLSM images were then used to quantify cell attachment and both width and thickness of cell pseudopods with Image J software.

2.3.3 Immunofluorescence Staining for Adhesion and Differentiation related proteins

The expressions of integrin $\beta 1$, vinculin, F-actin, osteocalcin (OCN), and osteopontin (OPN) were detected by a CLSM. Briefly, cells were fixed in 4% paraformaldehyde, rinsed with PBS, and permeabilized by 0.2% Triton-100 (Abcam, USA) for 15 min at room temperature after culture for 24, 48, and 96 h on disc-shaped Ti samples. Proteins mentioned above were immunostained with their antibodies as described herein [39]. Nuclei were counterstained with DAPI for 5 min. Samples were then rinsed and observed by a CLSM.

2.3.4 ALP activity assay

Cells were seeded and incubated for 48h on Ti discs in a 24-well plate. After cell lysis, ALP activity was detected in accordance with the instructions of the ALP activity detection kit (Beyotime, China). The wells without samples were set as the blank control. The amount of ALP was measured by absorbance at 405nm, and ALP activity

was defined as: $ALP\ (U/ml) = (A - A_{blank}) \times 0.665$. All experiments were carried out in triplicates.

2.3.5 Western blot

After culturing cells on Ti discs for 48h, cells were lysed in 0.1ml RIPA buffer (Millipore, USA) and centrifuged at 4°C, 2000r/min for 5 min to obtain supernatant. Proteins in the supernatant were separated by electrophoresis, transferred onto PVDF membrane at 200 mA for 1h, and blocked with 5% BSA (Abcam, USA) in the TBST solution (Abcam, USA). Proteins were further incubated with primary antibodies (anti-integrin β 1/vinculin/ALP/Runx-2/OPN, 1:500 dilution; internal control GAPDH, 1:3000 dilution) for 1.5h at room temperature and secondary antibodies (1:3000 dilution) for 1.5h at room temperature, respectively. The intensity of the protein bands was analyzed by UVP Chemiluminescence Imaging System (Labworks 4.6 software, USA).

2.3.6 RNA Extraction and Real-Time Polymerase Chain Reaction (PCR)

Cells were collected after culture on Ti discs for 24 and 48 h. Total RNA was extracted with TRIzol reagent (Thermo Fisher Scientific, USA). Reverse transcription was performed using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA). Real-Time PCR conditions were then set for 35 cycles, each cycle as 94°C/4 min, 94°C/20s, 60°C/30s, 72°C/30s (ABI Prism 7500, Seqgen, USA). RNA expression levels were determined for the following genes: vinculin, integrin β 1, Runx-2 and osteopontin. β -actin was used as the internal RNA control. Primer sequences of the genes were listed in Table S1.

2.4 Statistical analysis

SPSS 16.0 software was used to perform a one-way ANOVA, with post hoc analysis using Dunnett tests for multiple comparisons to determine the significant differences among the groups at significance level of $p < 0.05$. Data were presented as mean $\pm$ standard deviation (SD).

3. Results and discussion

3.1 Surface Properties of Materials

Surface properties of biological implants play an important role in cell-material interactions [40]. Several surface modification methods have been reported to modify Ti surfaces to produce a superior surface microstructure and roughness to attract osteoprogenitor cells and eventually increase mineralization on the implant surfaces [41,42]. In our study, a coating mainly composed of Ti, P and O elements is uniformly formed on the surface of Ti substrate, which is named TiP-Ti accordingly (Figure 1A). On these surfaces, hemispheric rosette-like “clumps” (20-40 μm in diameter), consisted of numerous nano-scaled “petals” growing radially outwards, are featured (Figure 1B) (more clearly in Figure S1). On the other surfaces, while sub-micron crystals formed by HA coating on HA-Ti samples, untreated Ti demonstrated a relatively smooth surface (Figure 1B). Surface roughness is one of the most important factors which significantly affects the biocompatibility of implants [43]. Several studies have reported that an increase in surface roughness, while superimposing both micron and submicron scale features on surfaces, led to enhanced osteoblast differentiation and production of local mediators of angiogenesis *in vitro* [44], increased bone-to-implant contact *in vivo* [45], and improved clinical rates of bone healing [46]. In particular, nano-scale roughness significantly increased both protein and cell membrane receptor production and further tuned osteoblast differentiation and tissue regeneration [47,48]. AFM analysis demonstrated that the treated surfaces have higher nano-scale surface roughness values (R_q) (HA-Ti: $88.53 \pm 54.64 \text{ nm}$, TiP-Ti: $97.96 \pm 49.13 \text{ nm}$) compared to untreated-Ti surface ($28.73 \pm 11.69 \text{ nm}$) (Figure 1C, Table 1). As for XRD characterization, high crystallinity of hydroxyapatite was detected on HA-Ti specimen surface. The chemical properties of calcium (Ca) and phosphorus (P) can be clearly identified. Moreover, obvious peaks attributable to $(\text{TiO})_2\text{P}_2\text{O}_7$, Ti and $\text{Ti}_3(\text{PO}_4)_4$ were present on TiP-Ti surfaces indicating P has been successfully introduced into the surface in crystalline state (Figure 2). It has been demonstrated that increased hydrophilicity on implant surfaces is more favorable for

protein adsorption and cell adhesion [49,50]. In this context, as shown in Figure 3, video S1 and S2, both HA-Ti and TiP-Ti surfaces became more hydrophilic after treatment since the contact angle was about 0° as opposed to $72.51 \pm 1.87^\circ$ on untreated-Ti surface. Subsequently, protein adsorption assay demonstrated that both TiP-Ti and HA-Ti surfaces were covered by more proteins than untreated-Ti surfaces (Figure S2A). In detail, the amount of protein adhered on TiP-Ti surfaces was twice of that on HA-Ti surfaces and four times of that on untreated-Ti surfaces (Figure S2B), informing that TiP-Ti is a superior candidate surface modification to improved cell adhesion.

Figure 1: (A) Analysis of the coating on TiP-Ti surface: *a and b*, SEM images with different magnifications; *c*, TEM image; *d*, EDS; *e*, XPS. (B) SEM images of implant surface at different magnifications. (C) AFM images of the surface topography of implant groups.

Figure 2: XRD patterns of the implant surfaces.

Table 1. Quantitative analysis of surface roughness by AFM (mean±SD)

Group	Ra (nm)	Rz (nm)	Rq (nm)
Untreated-Ti	16.78±9.23	168.82±9.01	28.73±11.69
HA-Ti	68.62±36.03*	732.51±327.63*	88.53±54.64*
TiP-Ti	75.29±29.03*	891.36±351.29*	97.96±49.13*

*Statistically significant difference compared with that of Untreated-Ti. Ra: Average Surface Roughness, Rz: Vertical Range, Rq: root-mean-square surface roughness.

3.2 *In vivo* animal study

In the biomechanical tests, TiP-Ti implants showed better intraosseous stability than HA-Ti and untreated-Ti implants: Both maximal push-out force and ultimate shear strength of TiP-Ti implants were significantly higher than those of HA-Ti and untreated-Ti implants after 4 and 12 weeks of implantation (Figure 3). More specifically, TiP-Ti group showed a significantly better improvement than HA-Ti group in either push-out force or shear strength; and enhanced push-out force or shear strength was detected in both HA-Ti and TiP-Ti groups compared to untreated-Ti group. Our findings in the biomechanical tests were further supported by both micro-CT and histological analysis. In the micro-CT analyses, bone volume ratio (BV/TV),

percentage osseointegration (%OI), and mean trabecular number (Tb.N) values of both HA-Ti and TiP-Ti groups were statistically significantly higher than those of untreated-Ti group after either 4 or 12 weeks. However, mean trabecular separation (Tb.Sp) of both HA-Ti and TiP-Ti groups was significantly less than that of untreated-Ti group. As for the comparison between TiP-Ti and HA-Ti, there was no significant difference in BV/TV, Tb.N, and Tb.Sp, while %OI of TiP-Ti were significantly higher than that of HA-Ti (Figure 4). Histological analyses of retracted samples demonstrated significantly increased bone area ratio (BA) and bone implant contact (BC) percentages in both HA-Ti and TiP-Ti groups compared to untreated-Ti group. Nevertheless, only BC of TiP-Ti group was significantly higher than that of HA-Ti in the histological analyses after 4 and 12 weeks of implantation (Figure 5).

It is commonly known that three main surface characteristics of the implants affecting osteointegration are surface energy, chemical composition, and surface morphology. In our study, both TiP-Ti and HA-Ti samples demonstrated similar surface energy and chemical compositions, as demonstrated in material characterization experiments (Figure 1Ad-e). Indeed, HA coating is more advantages in chemical composition since it contains not only phosphorus but also calcium, which is in favor of osteogenesis in theory [51–53]. However, interestingly, TiP-Ti group showed better initial stability and improved osteointegration than HA-Ti group. Thus, one can conclude that this could be because of the micro/nano-structured surface of TiP-Ti group, which helped to improve initial implant stability and osteointegration. In this study, we admitted the period of initial osteointegration as maximum 4 weeks after implantation. At this time point, maximum push-out force and ultimate shear strength values of TiP-Ti group were significantly higher than those of untreated-Ti and HA-Ti groups. Increased push-out force and ultimate shear strength of TiP-Ti implants suggested that osteointegration and cell bioactive reaction of TiP-Ti implant surfaces had a similar surface roughness value with our standard surface modification (HA-Ti), it is suggested that the biomimetic micro/nano-scaled hierarchical structure of TiP-Ti implant surface also contributed improving initial osteointegration in TiP-Ti group.

Figure 3: Biomechanical testing on ultimate shear strength and maximum push-out force at 4 and 12 weeks after implantation (* $p < 0.05$ vs Untreated-Ti; # $p < 0.05$ vs HA-Ti, $n=10$)

Figure 4: (A) Micro-CT images showing the volume of interest (VOI) and three-dimensional reconstruction screenshots on the transverse plane; (B) Quantitative analysis of osseointegration after implantation for 4 and 12 weeks (* $p < 0.05$ vs Untreated-Ti; # $p < 0.05$ vs HA-Ti, $n=20$)

Figure 5: (A) Histological micrographs of the section about 2 mm below the epiphyseal growth plate. (B) Quantitative evaluation of bone area ratio (BA%) and bone implant contact (BC%) of the Ti implant groups (* $p < 0.05$ vs Untreated-Ti; # $p < 0.05$ vs HA-Ti, $n=10$)

3.3 *In vitro* cell behavior

In vivo studies demonstrated that the biomimetic micro/nano-structured morphology enhanced osteointegration and intraosseous stability. To explore the mechanism, we further conducted *in vitro* experiments on proliferation, adhesion and osteogenic differentiation of BMSCs.

Cell viability on different samples from day 1 to day 9 was determined with an MTT assay. Cell viability in all implant groups was not different initially, but from day 3, the two modified groups showed significantly increased cell viability compared with untreated group. However, there was no significant difference of cell viability between TiP-Ti and HA-Ti in the whole period of culture (Figure S3A). Cell cycle progression was further examined at day 3 by FCM since the difference of cell viability rates emerged at this timepoint. In spite of significantly different surface morphology, both modified surfaces exhibited similar accumulation of cells in the *S* phase (Figure S3B).

We then observed morphology and pseudopodia of BMSCs in the initial adhesion period with both SEM and AFM. At 1h, BMSCs on untreated-Ti surface were globular with only a few filiform pseudopodia while the cells on HA-Ti surface already started to spread and cells on TiP-Ti surface were completely spread at the same time (Figure 6A). At 6h (Figure 6B), BMSCs on untreated-Ti were thin and very difficult to observe

in the SEM images. On the other hand, BMSCs on HA-Ti were well spread and many filopodia extensions were easily observed. However, the size of the cells was not consistent, and the floating filopodia informed weak contact with the surface. Cells on TiP-Ti surfaces were quite well spread and homogeneous and closely connected to each other by their intensive pseudopodia network. The pseudopodia of cells on TiP-Ti surface were obviously wider and thicker than those on HA-Ti. F-actin expression after 6h cultured on Ti implant surface was much higher on TiP-Ti surface than that of HA-Ti while both exceeded the untreated-Ti (Figure 6C). The quantitative analyses of cell morphology in the SEM and AFM images demonstrated that the cell attachment area percentage and tip-sample force values were significantly higher on TiP-Ti surface compared to both HA-Ti and untreated-Ti. While cell pseudopods were significantly thicker on Ti-P surfaces compared to those on the untreated-Ti surfaces, the width of cell pseudopods were higher than those on HA-Ti (Figure 6D). Taken together, there was an improved cell adhesion on the Ti-Ti surface compared to both untreated-Ti and HA-Ti.

Figure 6: (A) High-magnification SEM images and AFM topography of BMSCs after 1h culture

on Ti implant surface; **(B)** SEM images showing cell morphology on Ti implant surface as well as SEM and AFM measurements on the pseudopodia of BMSCs after 6h incubation; **(C)** CLSM images of BMSCs after 6h culture on the implants; **(D)** Quantitative evaluation of the SEM and AFM images (* $p < 0.05$ vs Untreated-Ti; # $p < 0.05$ vs HA-Ti, n=5)

Adhesion of BMSCs on the Ti surfaces was also investigated by visualizing integrin $\beta 1$, vinculin, and F-actin proteins with CLSM. Integrin $\beta 1$ and F-actin protein levels are remarkably increased in the BMSCs on TiP-Ti group compared to both HA-Ti and untreated-Ti groups after 24h culture. Similar trend was found in vinculin after 48h (Figure S4, Figure S5 and Figure S6). Late osteogenic differentiation of BMSCs was detected with immunostaining of OPN and OCN proteins after 96h culture (Figure S4 and Figure S7). Nevertheless, we did not observe any obvious difference between HA-Ti and TiP-Ti groups in both protein levels. Relative ALP activity of BMSCs, a surrogate marker of osteogenic differentiation, on both TiP-Ti and HA-Ti groups was significantly higher than untreated-Ti group from day 1 to day 7, but there was no significant difference between TiP-Ti and HA-Ti groups (Figure S8).

Adhesion and differentiation of BMSCs on Ti samples were further investigated by western blot and quantitative real-time PCR. Protein expression of integrin $\beta 1$ and vinculin by BMSCs cultured on TiP-Ti samples were significantly higher compared with those on HA-Ti samples at 48h. At the same time point, protein levels of both groups were significantly higher than that of untreated-Ti group. Runx-2, OPN, and ALP protein expression levels of BMSCs remarkably increased on both TiP-Ti and HA-Ti samples compared with untreated-Ti group. However, there was no statistical significance between TiP-Ti and HA-Ti samples (Figure S9). mRNA expressions of integrin $\beta 1$, vinculin, Runx-2, and OPN by BMSCs were significantly upregulated on TiP-Ti and HA-Ti samples compared to untreated-Ti group. Only integrin $\beta 1$ and vinculin expression levels of BMSCs on TiP-Ti group showed significantly higher levels than those on HA-Ti (Figure 7).

Figure 7: Quantitative real-time PCR analysis for mRNA expression levels. The relative rates of grey value were used for quantitative analysis. Results were displayed in terms of relative rates of GAPDH (* $p < 0.05$ vs Untreated-Ti; # $p < 0.05$ vs HA-Ti).

Both TiP-Ti and HA-Ti samples performed better than untreated-Ti samples in terms of adhesion, proliferation, and osteogenic differentiation of BMSCs. More importantly, TiP-Ti surfaces were superior or as good as HA-Ti on several biological parameters: First, TiP-Ti surfaces promoted cell adhesion better than HA-Ti surfaces. Second, MTT assay demonstrated no significant difference in the viability of the cells on both TiP-Ti and HA-Ti surface. Third, immunostaining of OCN and OPN, western blot analyses of ALP, Runx-2 and OPN, as well as qRT-PCR analyses of Runx-2 and OPN were also similar between TiP-Ti and HA-Ti surfaces. Therefore, one can conclude that increased osteointegration capacity of TiP-Ti compared to HA-Ti, did not stem from promoting proliferation or osteogenic differentiation of BMSCs, but mainly related to promoting cell adhesion. Indeed, our current findings on cell adhesion on TiP-Ti and HA-Ti surfaces supported our argument: In SEM images, cells on TiP-Ti surfaces were more obvious, wider and thicker than those on HA-Ti surfaces, which were also confirmed by the quantitative analysis of AFM. In CLSM images, F-actin, integrin $\beta 1$, and vinculin expressions by BMSCs were markedly promoted on TiP-Ti samples compared

with HA-Ti samples. In addition, western blot and qRT-PCR analyses of integrin β 1 and vinculin, cell adhesion proteins, further verified that TiP-Ti surfaces promoted cell adhesion better than HA-Ti surfaces.

To summarize, we propose that the hierarchical micro/nano-structured topography on TiP-Ti surfaces improved cell adhesion, promoted osteointegration, and eventually enhanced the implant stability in the early stages of peri-implant bone healing through the following mechanisms: Firstly, actin has been considered to be closely related to cell adhesion. Many studies reported that the regular and ordered surface topography could promote actin expression level of osteoblasts [54–56]. Moreover, surface roughness from 10nm to 10 μ m is the magnitude of biomacromolecules and osteoblasts, and therefore, from the biomimetics perspective, this magnitude of surface topography is in favor of initial adhesion of biomacromolecules and osteoblasts [48]. In this study, we designed a novel biomimetic micro/nano-structured surface with surface roughness value exactly in this scale. Many reports have also manifested that the gradient biomimetic morphology would be beneficial in promoting cell adhesion, especially initial adhesion [57–59]. Beyond that, on the surfaces with nano-scale surface roughness, it is reported that osteoblasts had more available adhesion sites, proliferated and interacted more, which eventually resulted in positive effect on osteogenesis [60–63]. Indeed, cells have a remarkable ability to sense topographical structure on implant surfaces and respond accordingly. In this context, because of its micro/nano-structured surface topography, TiP-Ti surface is a strong candidate implant surface to be used on titanium-based dental and orthopedic implants.

4. Conclusions

The novel hierarchically micro/nano structured topography of TiP-Ti surfaces demonstrated a significant improvement of osteointegration compared to HA-Ti, a standard surface modification. The advantage of TiP-Ti over HA-Ti surfaces was increased cell adhesion, since there was no significant difference in both proliferation and differentiation of BMSCs on these surfaces. Therefore, TiP-Ti surfaces contributed mainly to the promoted cell adhesion, especially initial adhesion, and eventually led to

a higher bone-implant binding rate and enhanced osteointegration, particularly early osteointegration. This special surface modification on Ti surfaces is a promising candidate surface modification to produce superior dental and orthopedic implants.

Data availability

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

Author Contributions

Nan Jang, Zhijun Guo, Li Zhang, and Songsong Zhu conceived ideas of this work. Zhijun Guo carried out all experimental work in relation to materials synthesis and characterization. Nan Jiang performed all biology-related work (cell and animal). All authors contributed to the analysis of data and result discussion as well as the writing of the manuscript.

Notes

The authors declare no competing financial interest.

Acknowledgement

This work was supported by the Natural Science Foundation of China (No.81470763, 81771097, 51673131) and the National Key Research and Development Program of China (2016YFA0201703/2016YFA0201700). We would like to thank Suilin Liu and Jiqui Wen (Analytical & Testing Center of Sichuan University) for the help of XPS and XRD testing, respectively.

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