Materials Science & Engineering C 82 (2018) 91–101

Contents lists available at ScienceDirect

Materials Science & Engineering C

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

Effect of crystalline phase changes in titania (TiO_2) nanotube coatings on platelet adhesion and activation

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ARTICLE INFO

Keywords:
Titania nanotubes
Crystalline phase
Platelet adhesion
Platelet activation

ABSTRACT

Objective: To explore the relationship between various crystalline phases of titania (TiO_2) nanotube (TNT) coatings and platelet adhesion and activation.

Methods: TNT coatings were fabricated on pure titanium foils by anodization and then randomly divided into four groups. Three groups were annealed at 350 °C, 450 °C and 550 °C in order to obtain different crystalline phases. The remaining group was not annealed and served as the control group. X-ray diffraction (XRD) was used to define the crystalline phases of different groups. Surface morphology, elemental composition, surface roughness, and contact angles were measured by scanning electron microscopy (SEM), energy dispersive spectroscopy (EDS), laser scanning confocal microscopy (LSCM) and contact angle analysis, respectively. Platelets were cultured on the TNT coatings for 30 min and 60 min to assess the number, viability, distribution, and morphology of the adhered platelets. CD62P fluorescence expression and the amount of released platelet-derived growth factor (PDGF) were detected to evaluate platelet activation.

Results: The un-annealed TNT coatings were amorphous and part of TNT converted to anatase after the 350 °C annealing treatment. The quantity of anatase increased upon annealing at 450 °C and transformed to rutile at 550 °C. Nanotubes of all four groups maintained a well-ordered structure, but the wall thickness of the nanotubes increased from (11.874 ± 1.660) nm for the un-annealed TNTs to (26.126 ± 2.130) nm for the 550 °C annealed TNTs. The surface roughness of the 550 °C annealed TNT coatings was the lowest and the water contact angle was the largest at (28.117 ± 1.182) °. The number and viability of adhered platelets after 30 min and 60 min were the highest on TNT coatings annealed at 450 °C. LSCM and SEM images revealed that the platelets that adhered on the 450 °C annealed TNT coatings aggregated, transformed, and spread most obviously. CD62P fluorescence expression results showed that the platelets on the 350 °C and 450 °C annealed TNT coating groups expressed the strongest fluorescence, followed by platelets on the 550 °C annealed group and the un-annealed group. The quantity of released PDGF was highest for the 450 °C annealed group at (4719 ± 86) pg/mL, and lowest for the un-annealed group at (4241 ± 74) pg/mL.

Conclusion: Crystalline TNT coatings encourage improved platelet adhesion and activation over amprphous analogues. The TNT coatings annealed at 450 °C resulted in the most improved platelet behavior. The TNT crystalline phase was the predominant influencing factor in platelet adhesion and activation.

1. Introduction

Dental titanium implants are widely used to replace missing teeth [1]. In order to achieve long-term success, it is necessary to obtain and maintain osseointegration between the implant surface and *de novo* bone [2–4]. The complex process of osseointegration is initiated upon blood contact during implant placement followed by the adsorption of plasma proteins, clot formation, osteogenic cell migration, and subsequent implant-cell interactions [5–8]. A variety of material modifications and surface treatments have been used to enhance the osseointegration of dental implants [4,9,10]. Similarly, pre-incubation of human whole blood on a titanium (Ti) implant surface enhances the thrombogenicity and the differentiation of human osteoblasts [6]. Thrombogenicity is considered very important for early implant healing

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http://dx.doi.org/10.1016/j.msec.2017.08.024
Received 19 March 2017; Received in revised form 7 July 2017; Accepted 9 August 2017
Available online 10 August 2017

and implant-blood interface integration is a prerequisite for osseointegration [11,12].

In the initial stage of implant healing, blood makes contact with the implant surface immediately, followed by the adsorption of plasma proteins and the mediation of platelet adhesion within a matter of a few seconds. Among blood cells, platelets are the first batch of cells to adhere on an implant surface and play a significant role during blood clotting, angiogenesis, and osteogenesis around the dental implant [6,12,13]. After adhering to the implant surface, platelets are activated to release bioactive factors (such as platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF)) to initiate the coagulation pathway for blood clot formation. The fibrin matrix of the newly formed clot acts as a bioengineering framework and facilitates the migration of mesenchymal stem cells (MSCs) from old bone to the implant surface. As such, blood clot formation around the implant surface plays an important role in osteoconduction and promotes contact osteogenesis [6,12–14]. PDGF and VEGF function as chemotactic factors and facilitate MSC migration, induce osteoblast proliferation, and differentiation. These growth factors can also improve early angiogenesis, bone matrix deposition, and bone formation [15–21]. These factors point to the importance of platelet adhesion and activation on the implant surface in initiating early healing around dental implants and osseointegration. Notably, platelet behavior is greatly influenced by implant surface properties [11,15,20–26].

Nanomaterials are promising biomedical materials with their unique physiochemical and structural characteristics. They have been widely used in biomedical areas, including nanomedicines, nanocarriers for gene delivery and drugs delivery [27–31]. With the development of nanotechnology, nanostructural modifications have been applied to implant surfaces with a view to improving osseointegration [32,33]. For instance, Cei et al. reported that titania (TiO_2) nanotube (TNT) coatings enhanced the quality and speed of bone formation [7]. Another study reported that TNT coatings enhance protein adsorption with faster adsorption of bovine serum albumin (BSA) and slower desorption [34]. Furthermore, the number of platelets that adhere on TNT coatings is significantly higher than on pure titanium surfaces, thus shortening the blood clot generation time [24]. Indeed, TNT coatings enhance cell adhesion, proliferation, and differentiation. Oh et al. reported that osteoblast proliferation on TNT coatings was 300–400% higher than on pure titanium after 48 h incubation [35]. Cells adhered on the TNT coatings and connected with each other through pseudopodia and extended to the inner walls of the nanotubes to increase cell-surface anchorage space [36]. Moreover, alkaline phosphatase (ALP) activity was 50% higher on the TNT coatings than the uncoated pure titanium analogues [37]. Enhanced expression of ALP, Col-I, and Osx genes was also found on TNT surfaces [38] with elevated levels of extracellular matrix secreted [39]. An *in vivo* study using domestic pigs also demonstrated that TNT coatings enhanced bone formation around implants [40].

Generally, TiO_2 nanotubes has three forms: amorphous, anatase and rutile. As-fabricated TiO_2 nanotubes at room temperature are usually amorphous. Through annealing at different temperatures, amorphous TiO_2 nanotubes were found to transform into anatase or mixed phase anatase/rutile crystalline forms [41,42]. Studies have shown that anatase and mixed anatase/rutile crystalline phases in TNT coatings can improve hydroxyapatite adsorption and the osteogenic cell response [16–18,42–45]. However, to date, there is little consensus on the optimal crystalline phase for the best cell response. Indeed, little attention has been given to the influence of TNT crystallinity on platelet behavior.

Inspired by the information outlined above, the aim of this study was to explore the influence of different TNT coating crystalline phases on platelet adhesion and activation and further understand the interrelationship between material crystalline phase, platelet behavior and biomaterial bio-activity. To this end, we fabricated TNT coatings on pure titanium surfaces with different crystalline phases through anodization and subsequent heat treatment and evaluated the platelet adhesion and activation on these various surfaces.

2. Materials and methods

2.1. Fabrication of TNT coatings

Titanium discs (n = 228; dimensions: 13.0 × 13.0 × 0.2 mm) were prepared from commercially pure titanium foil (99.6% purity, Qichen Material Corporation, Baoji, China). The discs were polished sequentially with *SiC* grinding papers (Struers, Denmark) from 200 to 1500 grit before being consecutively ultrasonically cleaned with acetone, ethyl alcohol, and deionized water before being dried in air. TNT coatings were fabricated on the surface of the pure titanium discs by anodization with a potentiostat (RNX 605D, Shenzhen Zhaoxin Electronic Instrument Equipment Co., LTD, China) at room temperature and 20 V for 30 min. A titanium disc was used as the anode and a copper sheet served as the counter electrode, with 0.15 mol/L NH_4F and 0.5 mol/L $(NH_4)_2SO_4$ in deionized water used as the electrolyte. A total of 228 samples were prepared. In order to obtain TNT coatings with different crystalline phases, the samples were divided into 4 groups (n = 57 for each group): un-annealed, annealed at 350 °C, 450 °C and 550 °C. The annealing procedures were conducted in a resistance furnace (SGM T40/10, SGM Equipment Company, Luoyang, China), at the three temperatures mentioned, in air, for 6 h, and at a heating rate of 5 °C/min.

2.2. Surface characterization

The crystalline phases of the samples were identified by X-ray diffraction (XRD, Bruker D8, Bruker, Germany) operating at a scan speed of 0.1 s/step in the 2-θ range of 20–60°. The surface morphologies of the TNT coatings were observed by scanning electron microscopy (SEM, Navo Nano SEM430, FEI, The Netherlands) at a voltage of 5 kV. The dimensions of the nanotubes were measured with Image Pro plus 6.0 software. Three fields from each sample were randomly selected and 20 nanotubes from each field were manually measured for the outer, inner diameter and wall thickness of the nanotubes. The chemical compositions of the TNT coatings were assessed through energy dispersive spectroscopy (EDS, FEI, the Netherlands), which was incorporated into the SEM system. Surface topography was observed and roughness was measured using laser scanning confocal microscopy (LSCM, LSM 700, Zeiss, Germany) through non-contact method. Contact angles were measured using a contact angle analyzer system (OCA15, Dataphysics, Germany), using a 2 μL drop of deionized water. Measurements were taken 10 s after the application of the drop.

2.3. Platelet preparation and incubation

This portion of the study protocol was approved by the Sun Yat-sen University Ethics Review Board. Human whole blood was collected from a young healthy volunteer with a vacuum blood collection tube containing 3.2% sodium citrate as an anticoagulation agent (BD Vacutainer, USA). To prepare platelet-rich plasma (PRP), the collected blood was centrifuged immediately at 200*g* for 10 min and the supernatant was collected. The supernatant was centrifuged again at 200*g* for 10 min and then the top three-quarters of the supernatant was removed and the rest of the plasma was mixed to yield PRP for immediate incubation. TNT coated samples were placed in 24-well plates (Corning, USA) and 200 μL PRP was added to each well. The 24-well plates were centrifuged at 150*g* for 10 min before being incubated on a horizontal shaker at 37 °C for further investigations.

2.4. Platelet counting and viability assessment

After incubation with PRP for 30 min and 60 min, the supernatants