

Research Article

Effect of Nanosheet Surface Structure of Titanium Alloys on Cell Differentiation

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Titanium alloys are the most frequently used dental implants partly because of the protective oxide coating that spontaneously forms on their surface. We fabricated titania nanosheet (TNS) structures on titanium surfaces by NaOH treatment to improve bone differentiation on titanium alloy implants. The cellular response to TNSs on Ti6Al4V alloy was investigated, and the ability of the modified surfaces to affect osteogenic differentiation of rat bone marrow cells and increase the success rate of titanium implants was evaluated. The nanoscale network structures formed by alkali etching markedly enhanced the functions of cell adhesion and osteogenesis-related gene expression of rat bone marrow cells. Other cell behaviors, such as proliferation, alkaline phosphatase activity, osteocalcin deposition, and mineralization, were also markedly increased in TNS-modified Ti6Al4V. Our results suggest that titanium implants modified with nanostructures promote osteogenic differentiation, which may improve the biointegration of these implants into the alveolar bone.

1. Introduction

There has been a concerted effort among materials scientists and clinicians worldwide to improve the performance of dental implants, with the aim of facilitating their integration into hard and soft tissues and/or extending their range of applications. The surface characteristics of an implant material affect its rate and extent of osseointegration [1]. Vandrovcová et al. [2] recently reviewed the growing evidence demonstrating that surface-modified materials are highly effective for adhesion, growth, and osteogenic differentiation of cells.

Osteogenic cells are known as anchorage-dependent cells. Accordingly, it is necessary to enhance early bone differentiation of mesenchymal cells and improve osseointegration on titanium surfaces [3–8]. A previous study showed that nanostructural modification can accelerate hard-tissue engineering through increased initial cell attachment to the surface [9].

Surface modification may allow the osseointegration period of any implant material to be shortened. Surface roughness has been shown to influence the initial cellular response, for example, the differentiation of human osteoblastic MG63 cells through $\alpha 5$ integrin interactions [10]. Another study found that the degree of osseointegration can be changed by controlling the size of nanostructures formed on titanium alloy surfaces [11].

In this study, nanostructures similar to the TiO₂ nanotubes formed by titanium deposition by TiO₂ sputtering [12] named titanium nanosheets (TNS) are formed on titanium alloy surfaces. Recently, it was shown that TiO₂ nanotube and TNS structures can be formed on titanium metal surfaces by treatment in 10 M NaOH aqueous solution at 30°C [13]. Treatment with NaOH aqueous solution is known to produce a rough, nanoscale surface [14]. A previous study reported that TNS produced by chemical processing promoted the

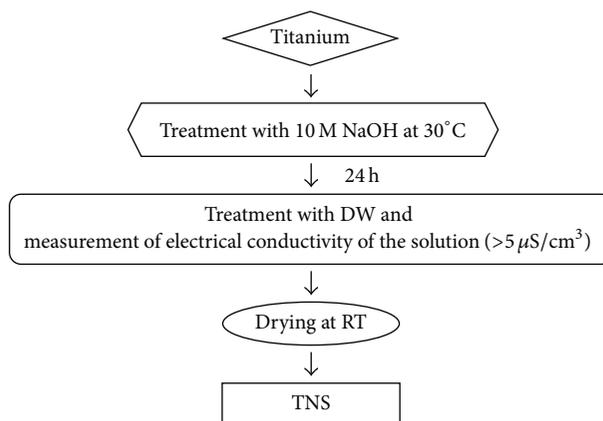


FIGURE 1: TNS deposition process.

osteogenic differentiation of rat bone marrow (RBM) cells [13]. The surface properties and structures of materials play important roles in the adsorption of proteins, which might influence cell behavior. However, the structure of TNS is unclear and the manner in which this structure influences bone differentiation must be evaluated for its application as an advanced implant material.

Titanium alloys are the most frequently used metallic materials for medical implants partly because of the protective oxide coating that spontaneously forms on their surface. Although titanium alloys exhibit excellent overall corrosion properties, metal ions released in physiological environments are still a concern. It has been suggested that the physicochemical and dielectric properties of the oxide film play important roles in determining implant biocompatibility [15]. Kim et al. [16] showed that NaOH treatment caused Ti-OH groups to form on the sodium titanate layer of the titanium surface. However, it is not yet clear how alloying species such as aluminum (Al) and V affect the bone differentiation of RBM cells following NaOH treatment.

The aims of the present study are to investigate the TNS structure of Ti6Al4V alloy and to evaluate the ability of this modified surface to affect osteogenic differentiation of RBM cells to increase the success rate of titanium implants. We prepare TNS structures on disks by NaOH treatment, investigate their morphology, and assess their biocompatibility.

2. Materials and Methods

2.1. Sample Preparation. Titanium alloy samples treated with NaOH to form TNSs on their surfaces were used as the experimental material. Unprocessed titanium alloy disks (Ti6Al4V) were used as the control group. Figure 1 outlines the process followed to produce TNSs. Ti6Al4V disks (15 mm diameter) were punched from sheets of 1 mm thick alloyed titanium (Daido Steel, Osaka, Japan). The disks were immersed in 10 M aq. NaOH and maintained at 30°C for 24 h. The solution in each flask was replaced with distilled water (200 mL) until the solution reached a conductivity of 5 $\mu\text{S}/\text{cm}$. Samples were then dried at room temperature.

2.2. Characterization of Materials. The surface topography of samples was qualitatively evaluated by scanning electron

microscopy (SEM, S-4000, Shimadzu, Kyoto, Japan) and scanning probe microscopy (SPM; SPM-9600, Shimadzu). The composition of the coating was analyzed by X-ray photoelectron spectroscopy (XPS; Kratos Analytical Axis ultra DLD electron spectrometer, Kratos Instruments, Manchester, UK) using a monochromatic Al $K\alpha$ X-ray source. Each sample was etched with argon ions for 2 min (evaporation rate 5 nm/min) to remove surface contaminants. Contact-angle measurements were performed using a video contact-angle measurement system (VSA 2500 XE, AST Products, Tokyo, Japan) at room temperature. Ultrapure water was used in contact-angle measurements.

2.3. Protein Adsorption Assay. Bovine serum albumin (BSA) fraction V (Pierce Biotechnology) was used as a model protein. Protein solution (300 μL , 1 mg/mL protein in saline) was pipetted onto each sample. After incubation for 1, 3, 6, or 24 h at 37°C, nonadherent proteins were removed and mixed with bicinchoninic acid (Pierce Biotechnology) at 37°C for 1 h. The amount of removed albumin and the total amount of albumin inoculated were quantified using a microplate reader at 562 nm. The rate of albumin adsorption was calculated as the percentage of albumin adsorbed on samples relative to the total amount of albumin in solution.

2.4. Cell Culture. RBM cells were isolated from the femurs of 7-week-old Sprague-Dawley rats. This study was performed under the Guidelines for Animal Experimentation at Osaka Dental University (Approval number 11-03038). Briefly, rats were euthanized using 4% isoflurane, and the bones were aseptically excised from the hind limbs. The proximal end of the femur and the distal end of the tibia were clipped. A 21-gauge needle (Terumo, Tokyo, Japan) was inserted into the hole in the knee joint of each bone, and the marrow was flushed from the shaft with culture medium (Eagle's minimal essential medium; Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Life Technologies Co., Carlsbad, CA, USA), penicillin (500 U/mL) (Cambrex Bio Science Walkersville Inc., Walkersville, MD, USA), streptomycin (500 $\mu\text{g}/\text{mL}$) (Cambrex Bio Science Walkersville Inc.), and Fungizone (1.25 $\mu\text{g}/\text{mL}$) (Cambrex Bio Science Walkersville Inc.).

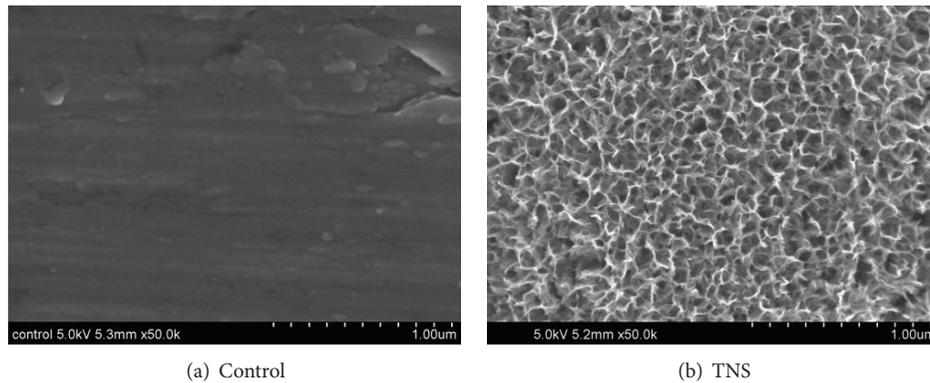


FIGURE 2: SEM images of the (a) untreated (control) and (b) treated (TNS) titanium alloy surface. The control sample exhibited relatively smooth surface features. A nanosheet structure formed upon NaOH treatment of the titanium alloy surface.

The resulting marrow pellet was dispersed by trituration, and the cell suspensions from all bones were combined in a centrifuge tube. RBM cells were cultured in 75 cm² culture flasks (Falcon, Becton Dickinson Labware, NJ, USA) in culture medium.

At confluence, cells were removed from flasks by trypsinization, washed twice with PBS, resuspended in culture medium, and seeded at a cell density of 4×10^4 cells/cm² into 24-well tissue culture plates (Falcon) containing test or control titanium disks. The cells were cultured at 37°C in a humidified 5% CO₂/95% air atmosphere.

2.5. Cell Adhesion and Proliferation. Cell adhesion was measured using the CellTiter-Blue Cell Viability Assay (Promega, Madison, WI, USA) according to the manufacturer's protocol. RBM cells were seeded on the samples at a density of 4×10^4 cells/cm² and allowed to attach for 1, 3, 6, 24, and 72 h. At each prescribed time point, nonadherent cells were removed by rinsing with PBS. CellTiter-Blue Reagent (50 µL) and PBS (250 µL) were then added to each well. After incubation at 37°C for 1 h, the solution was removed from the 24-well tissue culture plates (Falcon) and 100 µL was added to a new 96-well tissue culture plate (Falcon). The OD_{560/590} of the remaining solution was measured. The difference between the two optical densities was defined as the proliferation value.

2.6. Real-Time PCR Analysis. After 3 days of culture, the total RNA was extracted from the cells and cDNA was synthesized from 1 µg of RNA using a High-Capacity cDNA Archive Kit (Applied Biosystems Inc., Foster City, CA, USA). Runx2 mRNA expression was investigated by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) using a StepOne Plus Real-Time RT-PCR System (Applied Biosystems). To each well of a Fast 96-well Reaction Plate (0.1 mL well volume; Applied Biosystems) was added Taqman Fast Universal PCR Master Mix (10 µL), 1 µL of the primer probe set (20x Taqman Gene Expression Assays: Applera ordering number: Rss330360-m1Gadphs, Rss339198-m1runx2), sample cDNA (2 µL), and diethylpyrocarbonate-treated water (Nippongene, 7 µL). The plate was subjected to 40 reaction cycles of 95°C for 1 s and 60°C for 20 s. The reactive gene expression rate was calculated employing the $\Delta\Delta C_t$ method

[17] for each group assuming the gene expression rate of the negative control group.

2.7. ALP Activity. After 7 or 14 days of culture, cells were washed with PBS, lysed with Triton X-100 (Sigma, St. Louis, MO, USA, 0.2%, 200 µL), and the lysate was transferred to a microcentrifuge tube containing a 5 mm hardened steel ball. Tubes were agitated on a shaker (Mixer Mill Type MM 301, Retsh GmbH & Co., Haan, Germany) at 29 Hz for 20 s to homogenize each sample. ALP activity was measured using the Alkaline Phosphatase Luminometric ELISA Kit (Sigma) according to the manufacturer's protocol. The reaction was terminated with 3 N NaOH to a final concentration of 0.5 N NaOH and p-nitrophenol production was measured by absorbance at 405 nm using a 96-well microplate reader (SpectraMax M5, Molecular Device Inc., Sunnyvale, CA, USA). DNA content was measured using the PicoGreen dsDNA Assay Kit (Invitrogen) according to the manufacturer's protocol. To normalize ALP activity, the amount of ALP was normalized to the amount of DNA in the cell lysate.

2.8. Osteocalcin ELISA Analysis. The sandwich enzyme immunoassay used in this study is specific for rat osteocalcin (OCN) and can measure its levels directly in cell culture supernatant after 21 or 28 days of culture. The OCN levels in cell-culture supernatant were measured using a commercial ELISA kit (Rat Osteocalcin ELISA Kit DS, DS Pharma Biomedical Co., Ltd., Osaka, Japan) according to the manufacturer's instructions.

2.9. Mineralization. Calcium deposited in the extracellular matrix was measured after dissolution with 10% formic acid. The amount of calcium was quantified using a Calcium E-test Kit (Wako Pure Chemical Industrials Ltd.). After 21 or 28 days of culture, Calcium E-Test reagent (1 mL) and kit buffer (2 mL) were added to collected medium (50 µL), and the absorbance of the reaction products was measured at 610 nm using a 96-well microplate reader (SpectraMax M5). The concentration of calcium ions was calculated from the absorbance value relative to a standard curve.

2.10. Statistical Analysis. All experiments were performed in triplicate. All data are described as the mean \pm standard

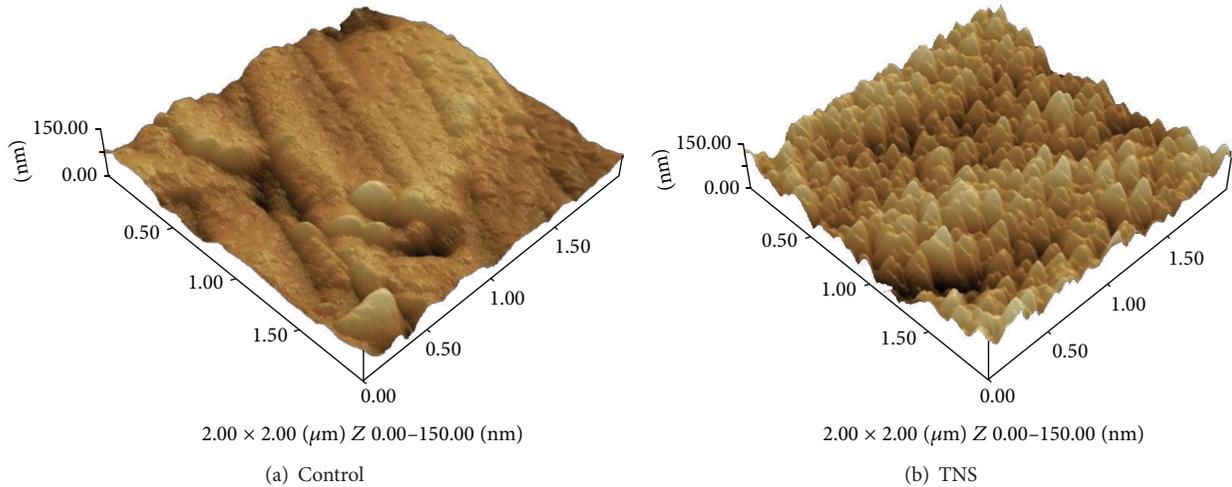


FIGURE 3: SPM images of (a) untreated (control) and (b) treated (TNS) titanium alloy samples.

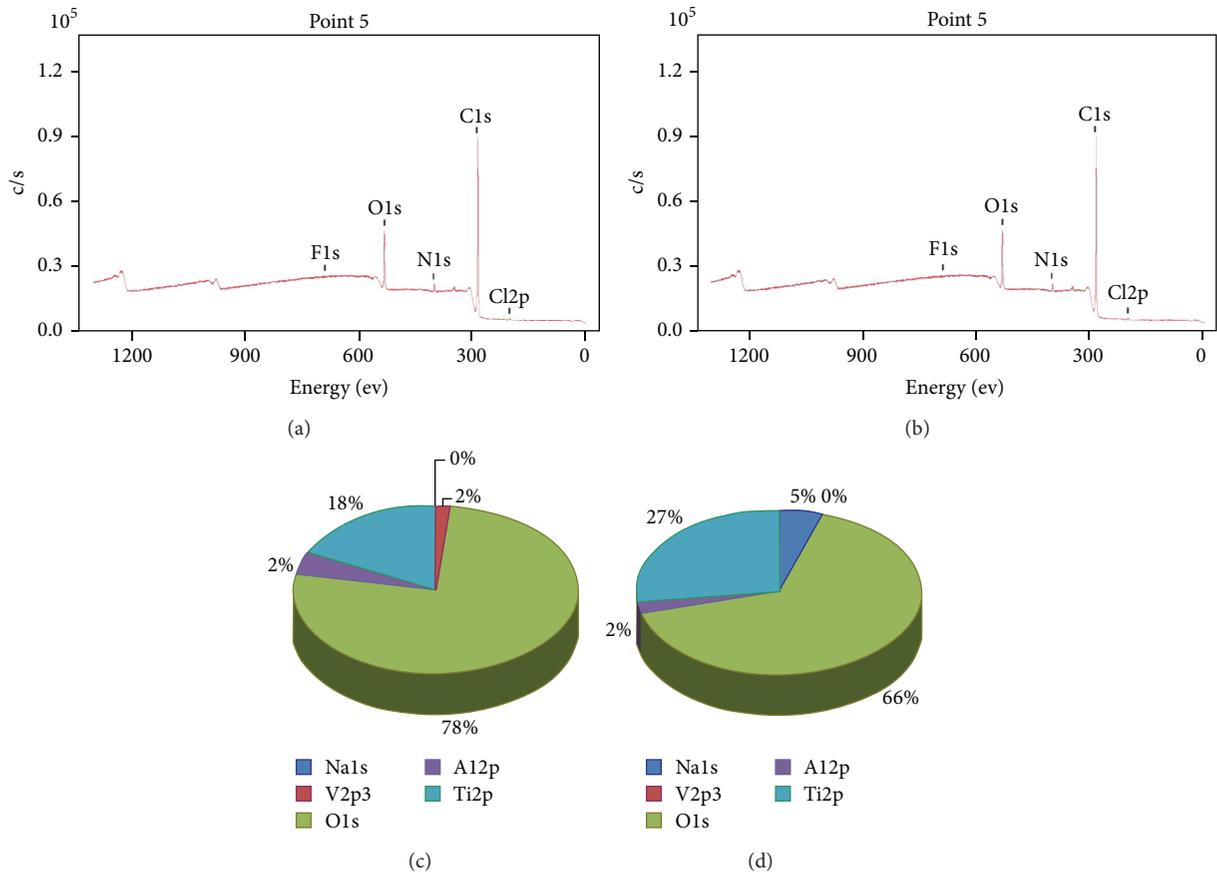


FIGURE 4: (a, b) Wide-range XPS images and (c, d) atomic concentration of (a, c) treated (TNS) and (b, d) control titanium alloy surfaces.

deviation. In all analyses, statistical significance was determined by paired two-tailed Student's *t*-test. A *P* value of 0.05 or less was considered to indicate statistical significance.

3. Results

3.1. Sample Preparation. SEM of the Ti6Al4V surfaces after modification in NaOH at 30°C revealed that a nanoscale

network structure formed. Figure 2 shows SEM images of the relatively smooth surface of the untreated Ti6Al4V and the nanoscale network structure of the chemically treated titanium alloy surface of the test group (labeled TNS), similar to that observed by Kasuga et al. [12]. The surface morphology and roughness of the TNS samples were examined by SPM; the results are shown in Figure 3. Porous network structures were observed in the treated samples. The surface roughness

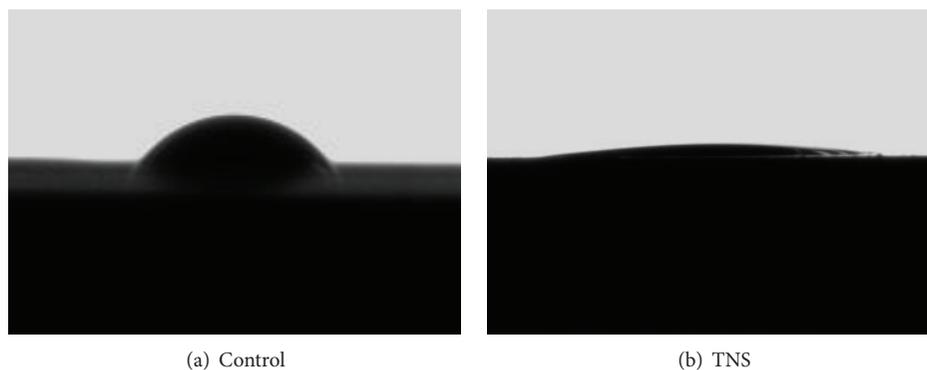


FIGURE 5: Cross-sectional view of a water droplet on the surface of (a) control and (b) treated (TNS) titanium alloy samples.

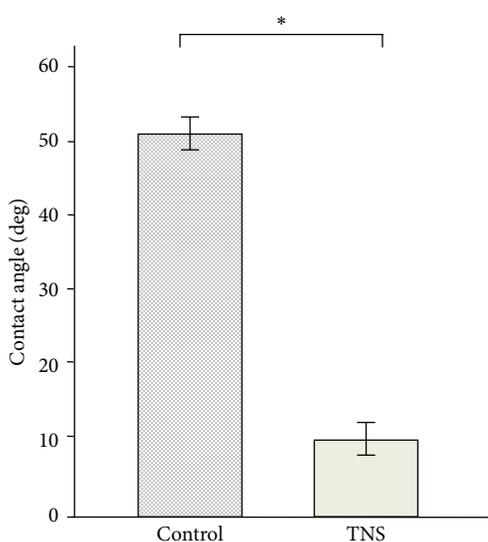


FIGURE 6: Contact angle of a water droplet on the surface of control and treated (TNS) titanium alloy samples.

(Ra) of the TNS and control groups was 19.9 and 47.8 nm, respectively. XPS revealed that Ti6Al4V released its alloying species of Al and V during NaOH treatment (Figures 4(c) and 4(d)). No V was detected on the modified surfaces of the treated alloy disks and screw post (Figures 4(a) and 4(b)). In contrast, the contents of Ti, Na, and O were hardly affected by alkali treatment. Cross-sectional views of water droplets on the surfaces of treated and control disks and their contact angles are depicted in Figure 5. A marked difference was found between the contact angles measured for the test and control disks (Figure 6).

3.2. Protein Adsorption. The amount of BSA protein adsorbed on the surface of the samples after 1, 3, 6, or 24 h of incubation was assayed (Figure 7). More protein was adsorbed onto the alkali-treated samples than the control ones.

3.3. Cell Adhesion and Proliferation. Cell adhesion as well as proliferation on the disks after 1, 3, 6, 24, and 72 h of

incubation was assessed (Figure 8). There were significant differences in cell adhesion and proliferation between alkali-treated and control samples after 1, 3, 6, 24, and 72 h.

3.4. Runx2 mRNA Expression. Runx2 is a transcription factor involved in the early stages of osteogenic differentiation. As shown in Figure 9, Runx2 activity was significantly higher in the test group using treated alloy with TNSs than in the control group after 3 days of culture.

3.5. Alkaline Phosphatase Activity. Cell differentiation was assessed by measuring the activity of the differentiation marker ALP in the test and control groups after 7 and 14 days. At both time points, ALP activity was significantly higher in the cells of the test group compared with that in the cells of the control group (Figure 10).

3.6. Osteocalcin Production. Figure 11 shows the production of OCN in the test and control groups after 21 and 28 days. The content of OCN in the supernatant of the test group was significantly higher than that in the supernatant of the control group.

3.7. Mineralization. Calcium deposition in the extracellular matrix of RBM cells in the test and control groups after 21 and 28 days is illustrated in Figure 12. Ca deposition was cumulative in the culture wells, so that measured levels normally increased with exposure time. Significantly more calcium was deposited by cells in the test group after both periods than by cells in the control group.

4. Discussion

This study investigated whether RBM cells responded differently to titanium alloy implants that had undergone chemical surface modification compared with those that had not. We found that the initial adhesion of cells and expression of the Runx2 transcription factor and RBM cell differentiation markers such as ALP and OCN were elevated in samples containing a TNS-modified titanium alloy disk compared with those containing an unmodified, polished titanium alloy disk. We also found that calcium deposition in the extracellular

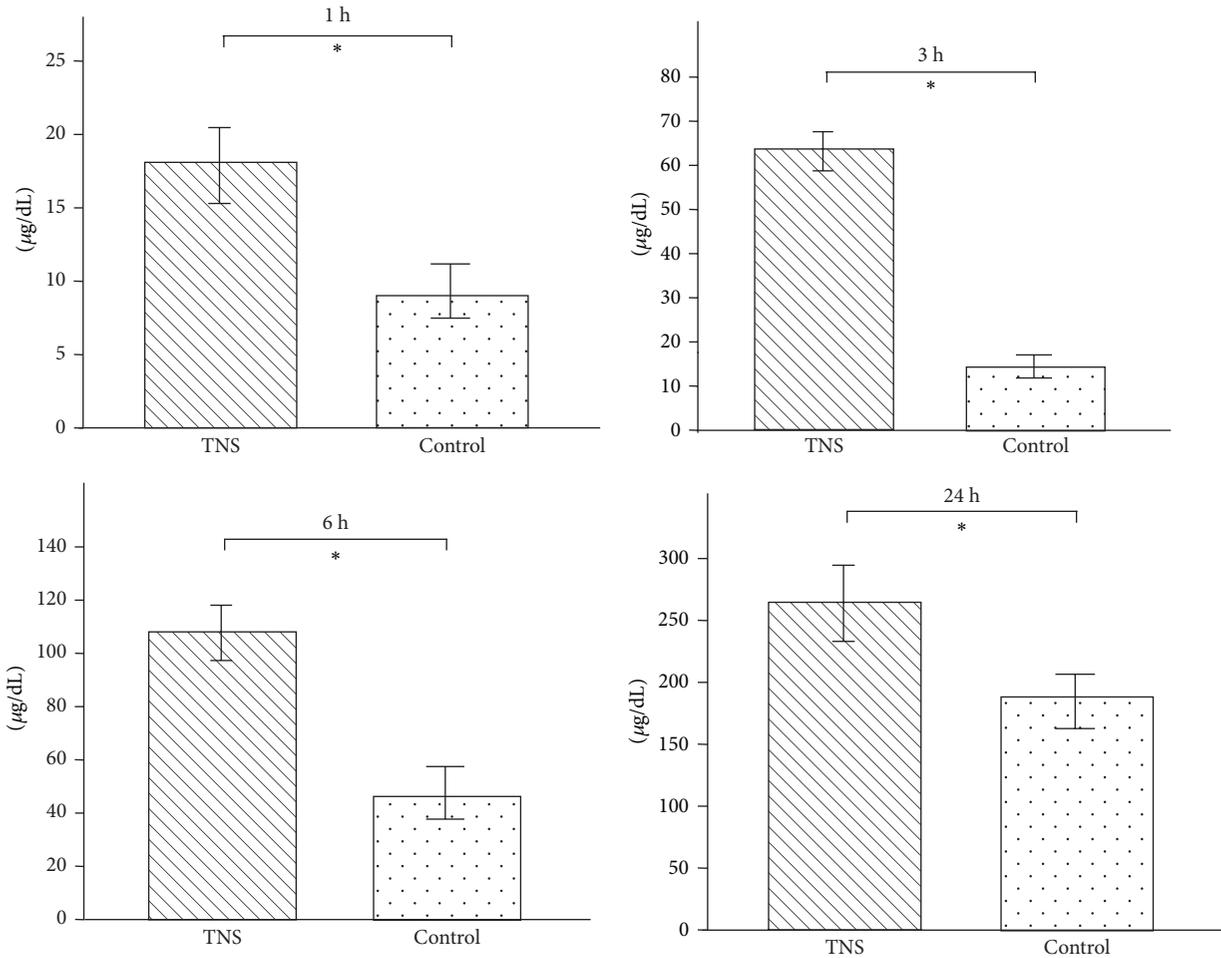


FIGURE 7: Initial adhesion of serum albumin to control and treated (TNS) titanium alloy samples.

matrix of the RBM cells was increased in the presence of a TNS-modified disk compared with that determined for the unmodified control disks. Our results suggest that a TNS structure on titanium alloy promotes RBM cell adhesion, differentiation, and activation, which augments calcium deposition.

The surface properties of dental implants have long been recognized as critical factors for achieving clinical success [18–21]. The topographical properties of nanostructures on titanium alloy surfaces play important roles in modulating cell responses at the implant-tissue interface, which can have a large effect on tissue integration with the implant [22]. Recently, we showed that TiO₂ nanotubes and TNSs could be formed on titanium metal surfaces by treatment in 10 M NaOH aqueous solution at 30°C [14], and we used this method here to prepare TNS-modified disks. Perla and Webster [14] suggested that TNSs on titanium surfaces lead to the regulation of osteogenic differentiation of bone marrow cells and enhance mineralization. Our results demonstrate that the TNS-modified titanium alloy disks were more hydrophilic and exhibited markedly improved wettability compared with unmodified ones. Better understanding of the surface roughness and topography of modified titanium alloy

surfaces is needed to assess their wettability. Ra is a commonly used height parameter to describe implant surface roughness. Ra of the TNS-modified titanium alloy surface was 19 nm, which was smaller than that of the untreated titanium alloy surface. The contact angles of the alkali-treated titanium alloy disks gradually decreased compared with those of the control group, which suggests that the wettability of the surface of the test group was decreased by NaOH treatment. Xing and Fujino and colleagues showed that a surface roughness between 13 and 16 nm was optimal for RBM cell culture [23, 24]. The nanonetwork structure formed on the titanium alloy disks here is similar to the hierarchical structure reported by Zhao and coworkers [25]. In their work, hierarchical nanotextured titanium alloy surface topographies with titania nanostructures that mimicked the hierarchical structure of bone tissues were produced by etching followed by anodization. Natural tissues are hierarchical structures of nanoscale building blocks assembled in an organized manner. Hierarchical structures composed of nanocomponents may provide a more suitable surface topography for bone marrow cell functions than simpler structures because they can better mimic the structure of natural tissues. In this study, XPS analysis revealed that Ti6Al4V released its alloying species of

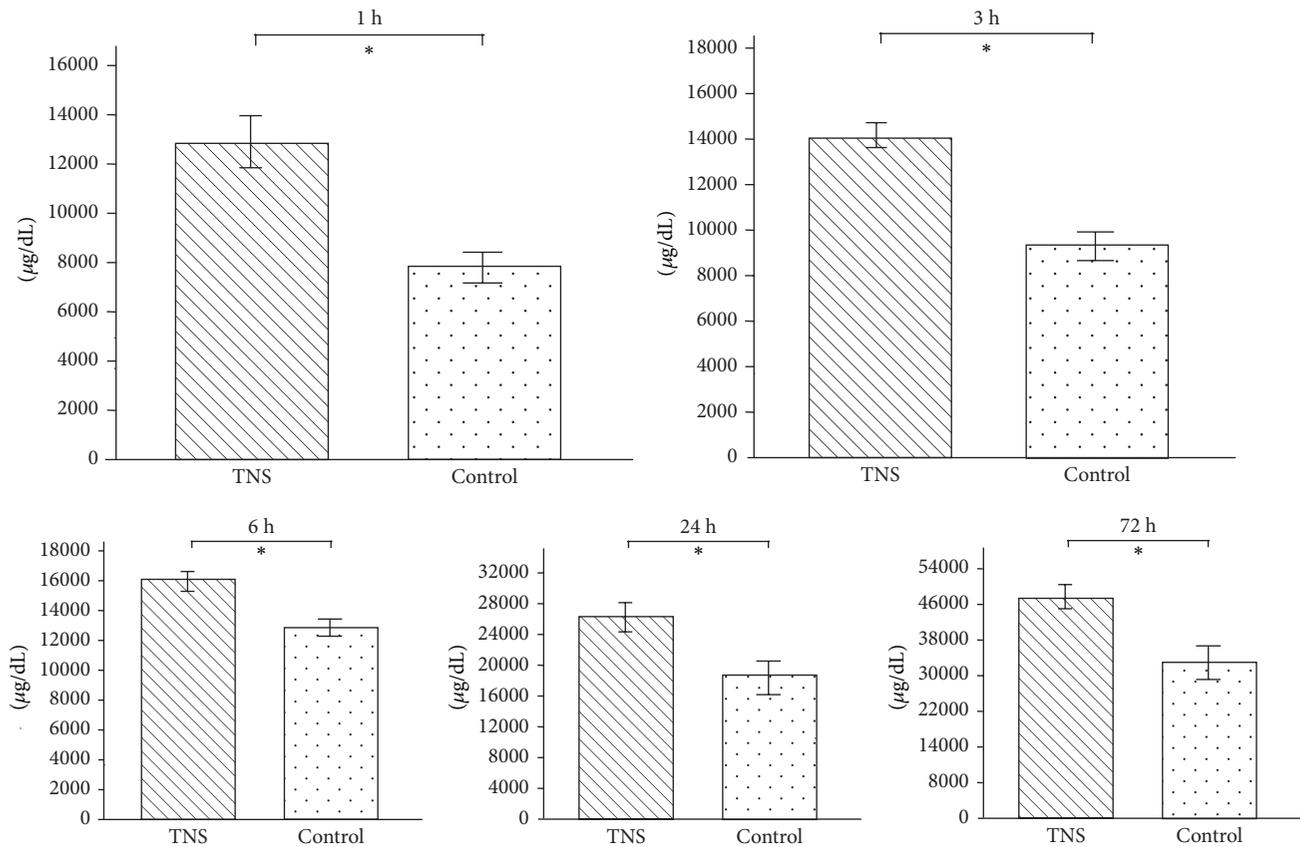


FIGURE 8: Initial adhesion of RBM cells to control and treated (TNS) titanium alloy samples.

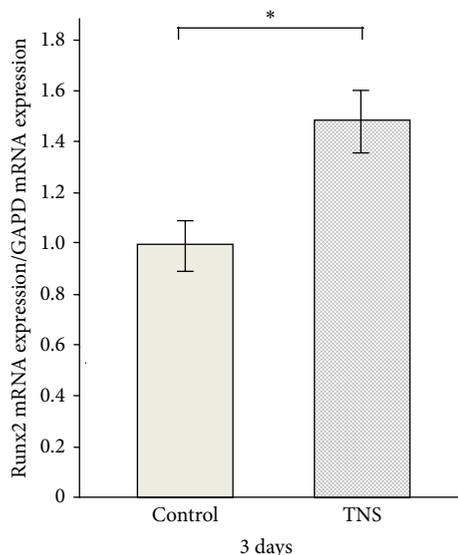


FIGURE 9: Expression of Runx-2 mRNA after 3 days of culture on treated (TNS) and control titanium alloy samples. There was a significant difference between the TNS and control groups after 3 days of culture ($P < 0.05$).

Al and V during NaOH treatment; no V was detected on the modified surfaces of treated alloy disks, whereas the contents of Ti, Na, and O were barely affected by alkali treatment. Our

research revealed that NaOH treatment led to the formation of a Ti-O-Na titanate layer on the titanium surface. V is known to exhibit biological toxicity, so it is interesting that no V was detected in the test group. It is considered that NaOH treatment caused a thick oxide film to form on the TiO₂ layer on the titanium alloy surface.

All implant surfaces are immediately covered with a layer of protein from the *in vitro* culture medium or *in vivo* biological fluids, and this interface modulates the cascade of cellular responses and behavior [26]. To examine the relationship between implant surface properties, opsonization, and phagocytosis under *in vivo* conditions, phagocytic experiments were conducted using a cell line in a cell culture medium that was supplemented with serum albumin and human opsonizing serum factors [27]. Albumin is the most abundant plasma protein and is known to discourage the adsorption of proteins that may stimulate inflammation and bacterial colonization [28]. In this study, adsorption of albumin by the test group was higher than that by the control group. The improvement of surface topography could be indirect; the adsorption of proteins or ions might act as a bridge between the nanostructured surface and cells.

Our results are the first comparison of RBM cell proliferation on TNS-modified titanium alloy surfaces with that on unprocessed controls. Surfaces with nanostructures are well known to possess increased surface area compared with those without such features [9, 29, 30]. This

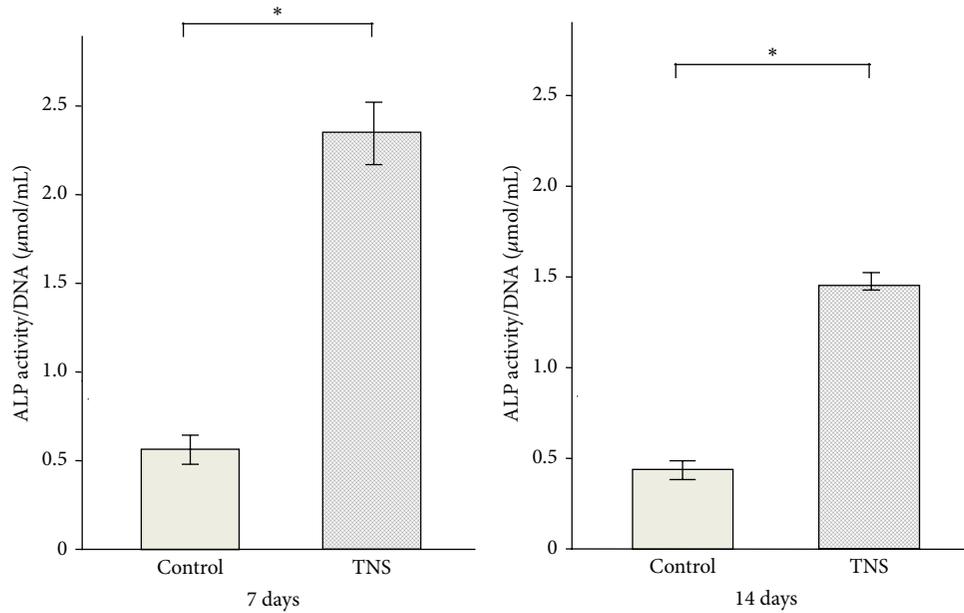


FIGURE 10: ALP activity after 7 and 14 days of culture on treated (TNS) and control titanium alloy samples. After 7 and 14 days, the ALP activity was expressed at significantly higher levels in the cells of the TNS samples compared with that of the control sample ($P < 0.05$).

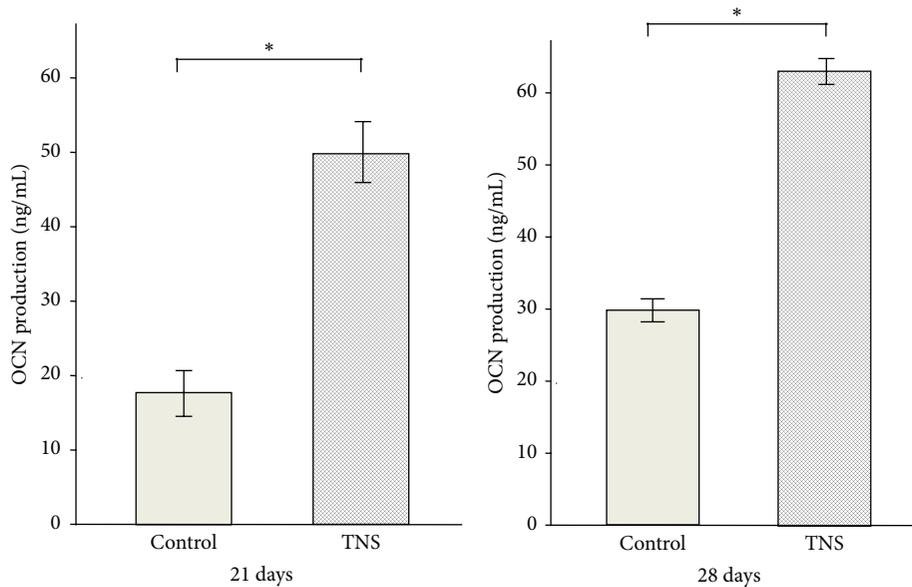


FIGURE 11: Osteocalcin production after 21 and 28 days of culture on treated (TNS) and control titanium alloy samples. The production of osteocalcin by cells of the TNS sample after 21 and 28 days was significantly higher than the cells of the control sample ($P < 0.05$).

increased surface area allows increased adhesion of cells such as osteoblasts and fibroblasts. Thus, the altered surface energies of materials with nanostructures have been suggested to promote tissue growth because of increased adsorption of select proteins compared with materials with microscale features [31]. Adsorption of select proteins can subsequently guide the adhesion of cells on the implant material surface, among other functions. Numerous studies have demonstrated improved cell adhesion and proliferation on

nanostructured surfaces that could be beneficial for various tissue applications, including bladder, bone, vasculature, and nervous systems [32–34]. A previous study indicated that TNSs on titanium surfaces helped to regulate the osteoblastic differentiation of bone marrow cells and enhance mineralization. In the present study, the TNSs formed nanonodules with a diameter of about 19 nm on the titanium surfaces, and these structures promoted the adhesion and/or proliferation of cells. The network structure of TNS on titanium alloy

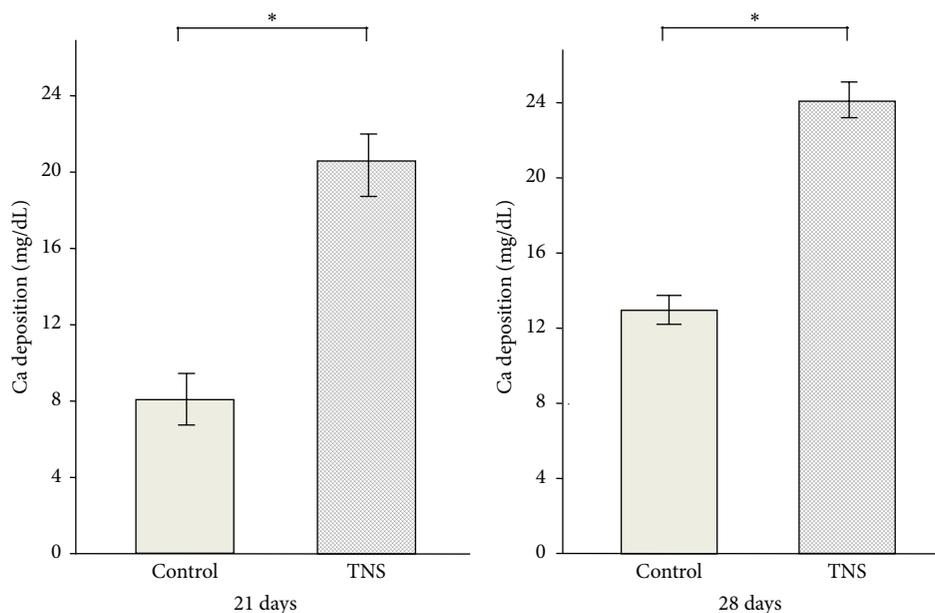


FIGURE 12: Calcium production after 21 and 28 days on treated (TNS) and control titanium alloy samples. The production of calcium by cells of the TNS sample after 21 and 28 days was significantly higher than that of cells of the control sample ($P < 0.05$).

facilitates rapid cell adhesion, spreading, and proliferation because of the mechanics of the TNS structure and chemical nature of the Ti-O-Na layer.

PCR analyses for the most specific and common markers of osteogenic induction were performed on RBM cells grown on treated and untreated titanium alloy implant materials. Different surface nanotopographies can alter the expression of important osteogenic regulatory genes such as Runx2. Runx2 mediates several early osteogenic gene responses for cellular adhesion [35]. Several trails of evidence show that surface modification leads to high expression of Runx2 mRNA [35–37]. The data from this study suggests that the elevated Runx2 mRNA expression in cells grown on the nanostructured titanium alloy surfaces compared with that on untreated surfaces is a causative factor in the differentiation of RBM cells into osteogenic cells.

ALP activity, OCN production, and calcium deposition were all elevated by the presence of TNSs on the implant surface. Importantly, the functional phenotypes expressed in the middle and late stages of culture, such as ALP activity and mineralization, were considerably increased. Substantial research has confirmed the ALP activation effect of surface-modified materials [14, 38–40]. Surface modification of implants has been found to affect ALP activation [14, 41, 42]; our results also support this conclusion. The observed increase of OCN production in the presence of a nanostructured surface is also in agreement with previous findings [14, 43].

Titanium and titanium alloy implants have become an essential treatment modality in reconstructive surgery in orthopedics and dentistry. However, patient morbidity and treatment complications need to be minimized, and outcome predictability and treatment indications should be maximized. Therefore, considerable effort has been expended

to developing new technologies to modify the surface of titanium to assist biointegration with bone [39]. The surface-modification method used here is effective and simple, involving incubation in NaOH at room temperature without a template [13]. The resulting TNS-modified titanium surfaces induce RBM cell bone differentiation.

5. Conclusion

In conclusion, our investigation of different implant surface nanostructures demonstrated that modifying the implant surface at the nanoscale leads to the regulation of osteogenic differentiation of bone marrow cells and enhances mineralization of the surface of titanium alloy. Further development of advanced implant materials using nanotechnology should improve their osseointegration.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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