Minocycline-Modified Pure Titanium Has Good Antibacterial Properties and Improves the Osteogenesis of Bone Marrow Stem Cells

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Research

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Abstract

Background : The development of an ideal implant material with appropriate antibacterial properties and that improves osteogenesis is essential for the guidance of new bone formation in orthopedic and tooth implant surgeries. In this study, we developed minocycline-modified pure titanium.

Methods: We exploited the chemistry of polydopamine (PD) for the coating of minocycline. PD was coated on pure titanium, on which minocycline was subsequently immobilized under certain conditions. Minocycline coating was verified by characterizing the surface chemical composition of the coated Ti sheet and was quantitatively measured by fluorescamine assay.

Results: The minocycline-coated pure titanium showed a lower bacterial adhesion rate and supported the spread of the osteogenesis differentiation of human mesenchymal stem cells (hMSCs). A remarkable increase in alkaline phosphatase activity and calcium deposition was found when hMSCs were cultured on minocycline-coated pure titanium for 28 days.

Conclusions: The minocycline-coated pure titanium may be optimized as clinically applicable bioactive materials for implant and bone materials.

Background

Pure titanium is widely used in surgical implant, orthopedic fixation, and tooth implant materials because of their good biocompatibility and corrosion resistance[1–3]. Implants are surrounded by cells, cytokines, and other parts of the marrow cavity with healing progress; parts of bone marrow stromal stem cells in the marrow cavity gradually differentiate into osteoblasts, which induce osteogenesis in the implant surface, form new bone tissue, and promote osteosynthesis[4–7]. However, infections on and around titanium implants remain a difficult problem. Implant-associated infection may lead to implant failure due to the formation of a bacterial surface biofilm[8–10]. Therefore, the antibacterial surface modification of Ti implants is important to prevent initial bacterial adhesion and promote bone bonding performance[11–14]. Minocycline is a second-generation semi-synthetic tetracycline broad-spectrum antibiotics, and its antibacterial spectrum is similar to that of tetracycline with a high degree of lipophilicity, strong tissue penetration, and good antibacterial effect. Minocycline is widely used in the clinical treatment of acne, non-gonococcal urethritis, and periodontitis. It specifically binds to the 305 subunit of ribosome and prevents aminophthalein tRNA from entering the site. Thus, it prevents the extension of the peptide chain, inhibits the protein synthesis of the pathogen, and plays an antibacterial role. The drug is bacteriostatic at low concentration and has bactericidal effect at high concentration. Its antibacterial spectrum includes Gram-positive and -negative bacteria, such as Rickettsia, Mycoplasma, Chlamydia, Spirochetes, Mycobacterium, Plasmodium falciparum, and Toxoplasma[15–18].

In this study, we developed a minocycline-coated pure titanium as an antibacterial material and a guidance material for bone regeneration. We used polydopamine (PD) as the coating medium for

minocycline coating[19, 20]. The objectives of this study were to evaluate the antibacterial properties of minocycline-coated pure titanium and to investigate its effects on the viability and osteogenic differentiation of human mesenchymal stem cells (hMSCs).

Methods

Materials

Titanium (Ti) sheets (>99.9% pure) were purchased from Northwest Institute Metal Research (Xi'an, China). Tris-HCl buffer was purchased from Shelton Scientific, Inc. (Peosta, IA, USA). Dulbecco's modified eagle medium with low glucose, phosphate buffered saline (PBS), trypsin–ethylenediaminetetraacetic acid solution, and penicillin–streptomycin solution were purchased from Gibco Bio-Rad Laboratories (Rockville, MD, USA). Fetal bovine serum (FBS) was purchased from Wisent (Montreal, Canada).

Preparation and characterization of titanium coated with minocycline

Ti sheets were ground and polished to a mirror surface and ultrasonically cleaned in acetone, ethanol, and distilled water for 15 min each. Ti sheets were immersed in dopamine hydrochloride solution (2 mg/mL; 10 mM Tris-HCl buffer; pH 8.5) and stirred at 50 rpm for 4 h on an orbital shaker. Samples were thoroughly rinsed and stirred in distilled water overnight. PD-coated Ti sheets were immersed in minocycline solution (100, 200, and 500 mg/mL; 10 mM Tris-HCl buffer; pH 8.5) and incubated at 37 °C overnight.

Minocycline on coated Ti sheets (MHCl-Ti) was quantified as follows. Supernatant (75 mL) retrieved from the sample after coating was placed in a 96-well plate and reacted with 25 mL of fluorescamine solution (100 mg/mL in acetone) for 1 min at room temperature. The fluorescence intensity of the sample (480 nm excitation wavelength and 520 nm emission wavelength) was measured using a spectrometer (SpectraMAX M2e, Molecular Device, Sunnyvale, CA, USA). The actual concentration of minocycline in each sample was compared with the fluorescence intensity of minocycline standards of known concentration (500 mg/mL). The amount of immobilized minocycline was calculated by excluding the amount of unreacted minocycline in the supernatant. The Ti sheets coated with minocycline were incubated in 70% ethanol for 15 min under UV light and rinsed with PBS thrice. Magnified images of the Ti sheets were obtained by scanning electron microscopy (SEM) (JSM-6701F, JEOL, Tokyo, Japan). All samples were completely dried in a desiccator at room temperature for 24 h and then coated with gold. X-ray photoelectron spectroscopy (XPS) (ESCA LAB 2001, Thermo VG Scientific, MA, USA) was used to analyze the surface chemical composition. The differences in the chemical composition of each sample were analyzed by using a peak-fitting software.

Bacterial adhesion and evaluation

Samples were coated via incubation in 24-well plate with 2 mL of sterile artificial saliva (0.33 g/L KH_2PO_4, 0.34 g/L NaH_2PO_4, 1.27 g/L KCl, 0.16 g/L NH_4Cl, 0.58 g/L NaCl, 0.17 g/L $CaCl_2{\cdot}2H_2O$, 0.6 g/L

NaSCN, 0.2 g/L urea, 0.03 g/L glucose, 0.002 g/L vitamin C, and 2.7 g/L mucin [M-1778]) for 4 h at 37 °C. Then, polished Ti and MHCl-Ti sheets were incubated in 2 mL of M-BHI medium containing 30 µL of *Streptococcus mutans* culture at 37 °C under anaerobic conditions. The medium was replaced with an equal amount of fresh sterile medium every 2 days. Separate samples were removed from the medium after 7 days for biofilm evaluation. Samples were fixed in 2.5% glutaraldehyde in 0.1 M PBS with pH 7.4 for 2 h. Then, the samples were dehydrated with increasing concentrations of ethanol solutions (50%, 70%, 80%, 90%, and 100%) and air-dried. Magnified images of the Ti sheets were obtained through SEM. The samples were coated with 20–30 nm of gold before examination. The adhering bacteria were stained with LIVE/DEAD BacLight bacterial viability kit (L7007, Invitrogen, USA). Fluorescein diacetate solution (2 µL) and ethidium bromide (2 µL) were mixed in 1 mL of cold distilled water, and 200 µL of the final mixture was dropped onto the sampled plaque and incubated for 15 min at 0 °C. Observations of the fixed biofilm were then carried out on LSM510 (Carl Zeiss, Germany).

hMSC culture and osteogenic differentiation

hMSCs were seeded at 5 × 104 cells/well on polished Ti and MHCl-Ti (200 mg/mL group) sheets in 24-well culture plates. Samples were sterilized with 70% ethanol, washed with sterile PBS twice, and irradiated by short-wavelength UV light for 30 min. The hMSCs were cultured in normal culture medium for 1 day and then in an osteogenic medium (alpha minimum essential medium containing 15% FBS, 1% antibiotic/antimycotic solution, 10 mM sodium β-glycerol phosphate, 50 µg/mL L-ascorbic acid, and 10^{-7} M dexamethasone). Media were replenished every 48 h for up to 28 days.

MTT assay MTT was used to assess cell viability on experimental (MHCl-Ti 200 mg/mL) and control (Ti) substrates at 1, 4, and 7 days. Samples were washed with PBS and incubated in media supplemented with 40 µL of 5 mg/mL MTT. Purple MTT formazan crystals formed in 4 h were dissolved by adding 400 µL of dimethyl sulfoxide, and optical density at 570 nm was measured in a separate 96-well microplate using 100 µL of aliquots.

Alkaline phosphatase (ALP) activity Samples were incubated at 25 °C for 30 min with 400 µL of p-nitrophenyl phosphate (Thermo). Total protein from the cultured cells was extracted by 95% M-PER protein extraction kit (Thermo) with 5% protease inhibitor cocktail (Thermo). Reactions were terminated with 200 µL of 2 M NaOH, and absorbance was measured at 405 nm to determine the release of p-nitrophenol. Enzymatic activity was probed after osteogenic induction at days 7, 14, 21, and 28.

Alizarin red staining Samples were stained with Alizarin red-S at days 7, 14, 21, and 28 after osteogenic induction to detect the calcium deposited by the cells. First, the samples were fixed with ice-cold ethanol for 1 h, and then, they were treated with 40 nM Alizarin red-S solution (pH 4.2) for 30 min. The samples were thoroughly washed with water. Alizarin red-bound calcium was extracted using 10 wt.% cetylpyridinium chloride in 10 mM sodium phosphate (pH 7.0) for 20 min at room temperature. The concentration of calcium was measured in a separate 96-well microplate using 100 µL of aliquots at 570 nm.

Results

Characterization of MHCl-Ti

The amount of minocycline coated on Ti sheets increased with increasing concentration. Approximately 1.9 mg minocycline was immobilized on 1.0 cm^2 substrate reacted with 200 mg/mL of minocycline and was remarkably greater than that immobilized in the 100 mg/mL group (Fig. 1A). SEM images of the surface of Ti and MHCl-Ti showed similar morphology (Fig. 1B). The surface chemical composition of the fibers was analyzed through XPS. As shown in Fig. 1a, the intensity of the N1s (399 eV) peak on the surface of MHCl-Ti was higher than that on Ti. The appearance of N1s and Ti–O–N peaks indicated that PD and minocycline were successfully coated (Fig. 1C).

Bacterial adhesion on polished Ti and MHCl-Ti

SEM images of the bacterial biofilm on Ti and MHCl-Ti surfaces as a function of exposure time are shown in Fig. 2. Large-sized bacterial clusters were observed on the surfaces after 7 days of exposure (Figs. 2A–D). However, the attached bacteria on Ti surface (Fig. 2A) were relatively more compared with those on MHCl-Ti (Figs. 2B–D). CLSM revealed an obviously vital (green) bacterial layer on surface of Ti and a dead (red) microorganism layer on the surface of MHCl-Ti (Fig. 3A), and MHCl-Ti (200 mg/mL group) was wholly red in color and showed the highest antibacterial properties among the samples (Fig. 3C).

Viability and osteogenic differentiation of hMSCs on polished Ti and MHCl-Ti

The cell morphology of hMSCs cultured on different substrates for 1 day in osteogenic medium was characterized by SEM. The hMSCs showed good cell adhesion and spreading at day 1 (Fig. 4A). MHCl-Ti was more biocompatible, as evaluated by the comparable viability levels at days 1, 4, and 7 (Fig. 4B). ALP activity and the amount of calcium were measured to examine the osteogenic differentiation of hMSCs (Figs. 4C and D). The ALP activity of hMSCs cultured on MHCl-Ti was remarkably higher than that on Ti at days 21 and 28. The amount of mineralized calcium in hMSCs cultured on MHCl-Ti was considerably higher compared with that on Ti at days 21 and 28.

Discussion

Titanium implants are widely used in clinical orthopedic materials, dental implants are one of them[21].However, the use of titanium implants can lead to implant-related infections, leading to the loosening and even falling off of implants. The internal environment of oral cavity is especially suitable for the colonization and reproduction of pathogenic microorganisms, which leads to the decrease of bone binding ability and healing speed of bone tissue around dental implants, and the destruction of stability of surrounding soft tissue, which further affects the curative effect of implant repair.The stability around the implant depends on the stability of the surrounding bone tissue and soft tissue, especially the stability of bone tissue, which mainly depends on the binding process of the surrounding bone

tissue[22].Therefore, the bone tissue absorption around the implant is closely related to the repair effect.To solve this problem, the implant was modified by surface functionalization to achieve antibacterial and enhance the ability of bone bonding.In this study, we developed a minocycline-coated pure titanium as an antibacterial material and a guidance material for bone regeneration. The minocycline-coated pure titanium showed a lower bacterial adhesion rate and supported the spread of the osteogenesis differentiation of human mesenchymal stem cells (hMSCs). A remarkable increase in alkaline phosphatase activity and calcium deposition was found when hMSCs were cultured on minocycline-coated pure titanium for 28 days. We believe that this material is an ideal material for bone repair and dental implant. Further research should focus on the bone-binding and antibacterial properties of the material in vivo

Conclusions

We developed minocycline-coated titanium through a simple coating method. We obtained the chemical composition of the surface of titanium with minocycline coating via XPS. Minocycline-coated titanium reduced bacterial survival and enhanced the osteogenic differentiation of hMSCs. Therefore, we conclude that minocycline-coated titanium can be used as tooth implant and bone fixation materials.

Abbreviations

PD:polydopamine ; hMSCs:human mesenchymal stem cells; Ti:Titanium; PBS:phosphate buffered saline; FBS:Fetal bovine serum; SEM:scanning electron microscopy; XPS:X-ray photoelectron spectroscopy; ALP:Alkaline phosphatase

Declarations

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZY conceived of the study and participated in its design and coordination,
and helped to perform experiments and analysis as well as draft the manuscript. DL participated in performing experiments and data analysis as well as drafting the manuscript. ZFconceived of and designed the study and participated in analysis and
interpretation of data as well as manuscript review and final calibration.All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval

Not applicable.

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Figures

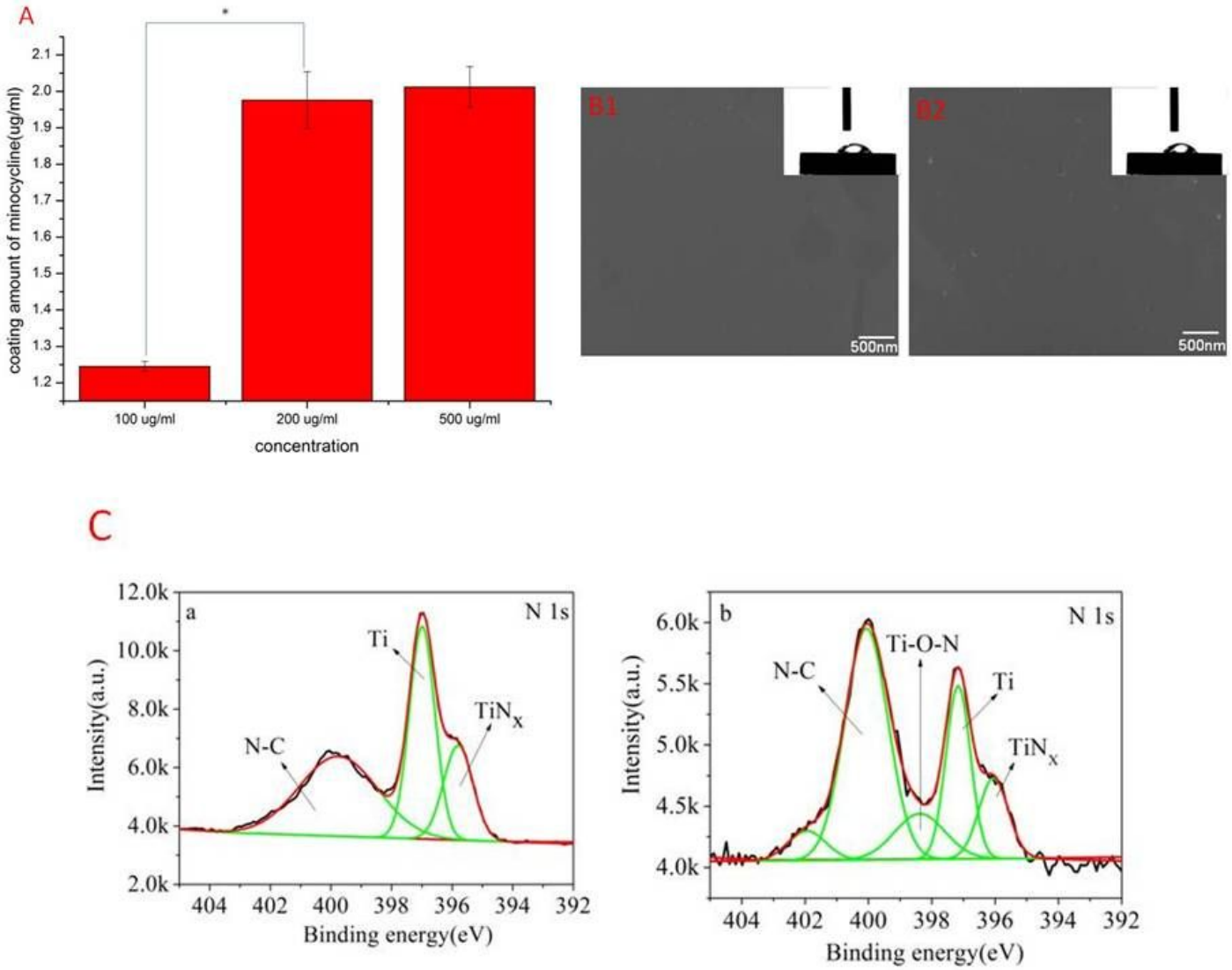

Figure 1

(A) Amount of minocycline immobilized on the samples measured by fluorescamine assay. (*P < 0.01) (B) SEM micrographs of the samples. (B1) Ti, (B2). MHCl-Ti. (C) Surface chemical composition of the samples, as examined by XPS. (a) Ti, (b) MHCl-Ti.

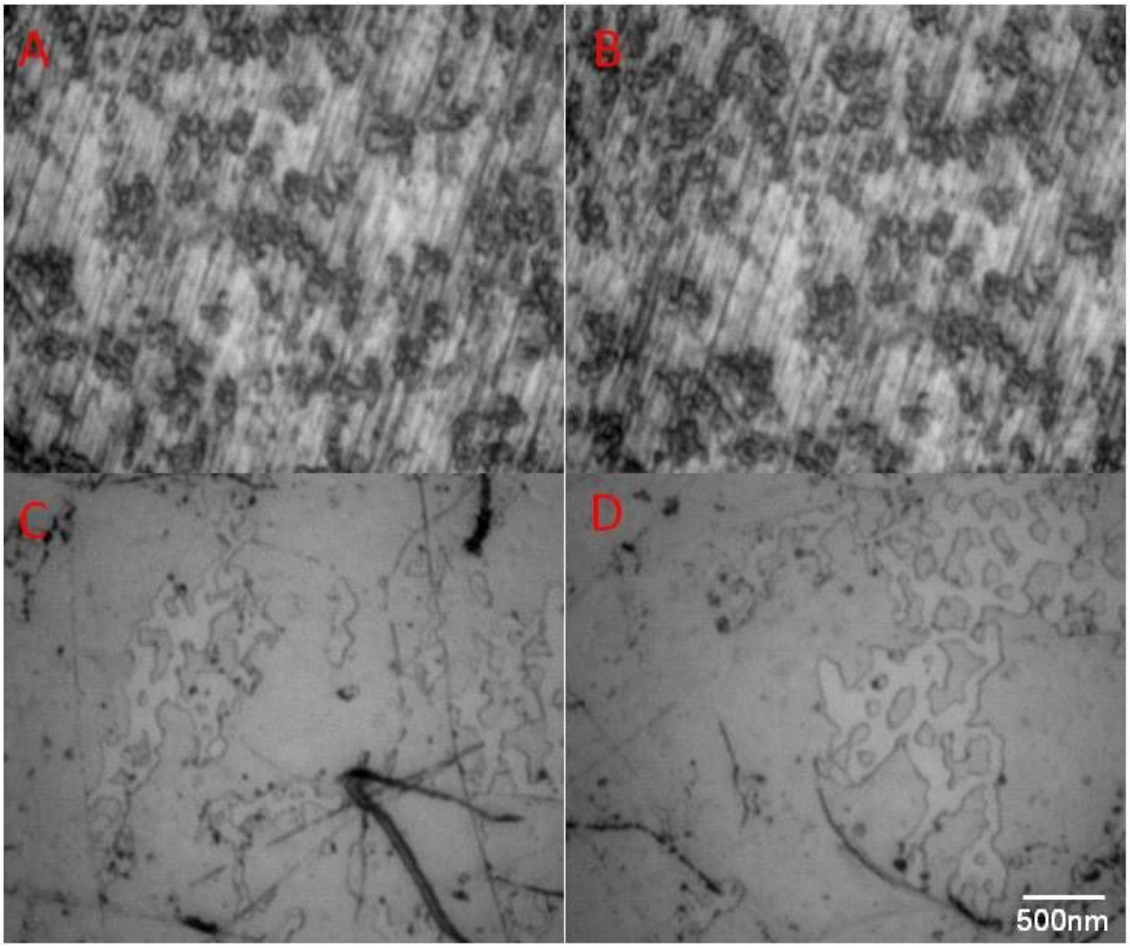

Figure 2

SEM micrographs of S. mutans cultured on samples for 7 days. (A) Ti; (B, C, and D) MHCl-Ti (100, 200, and 500 μg/mL, respectively).

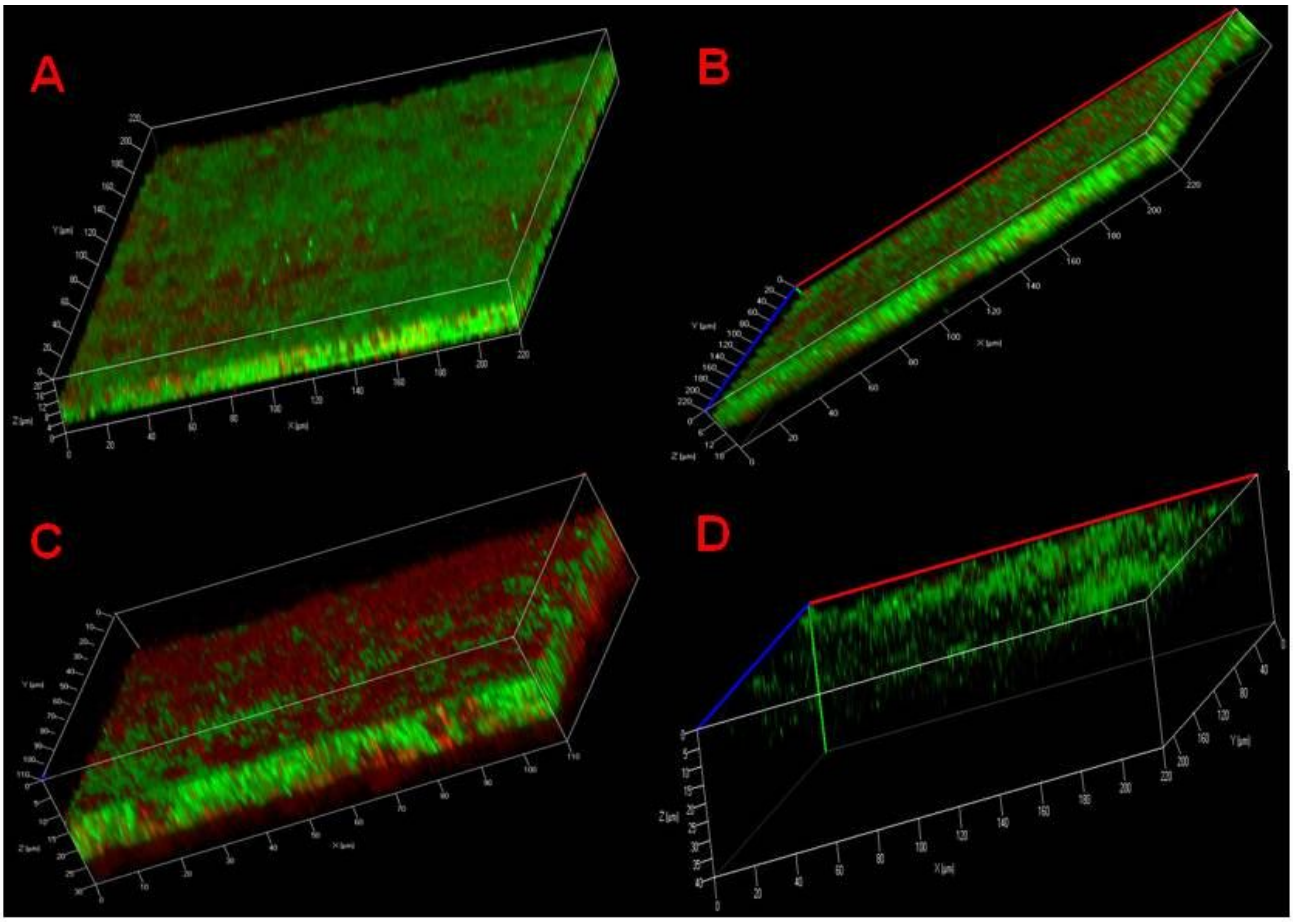

Figure 3

Confocal fluorescence microscopy of S. mutans cultured on different substrates for 7 days. (A) Ti; (B, C, and D) MHCl-Ti (100, 200, and 500 μg/mL, respectively).

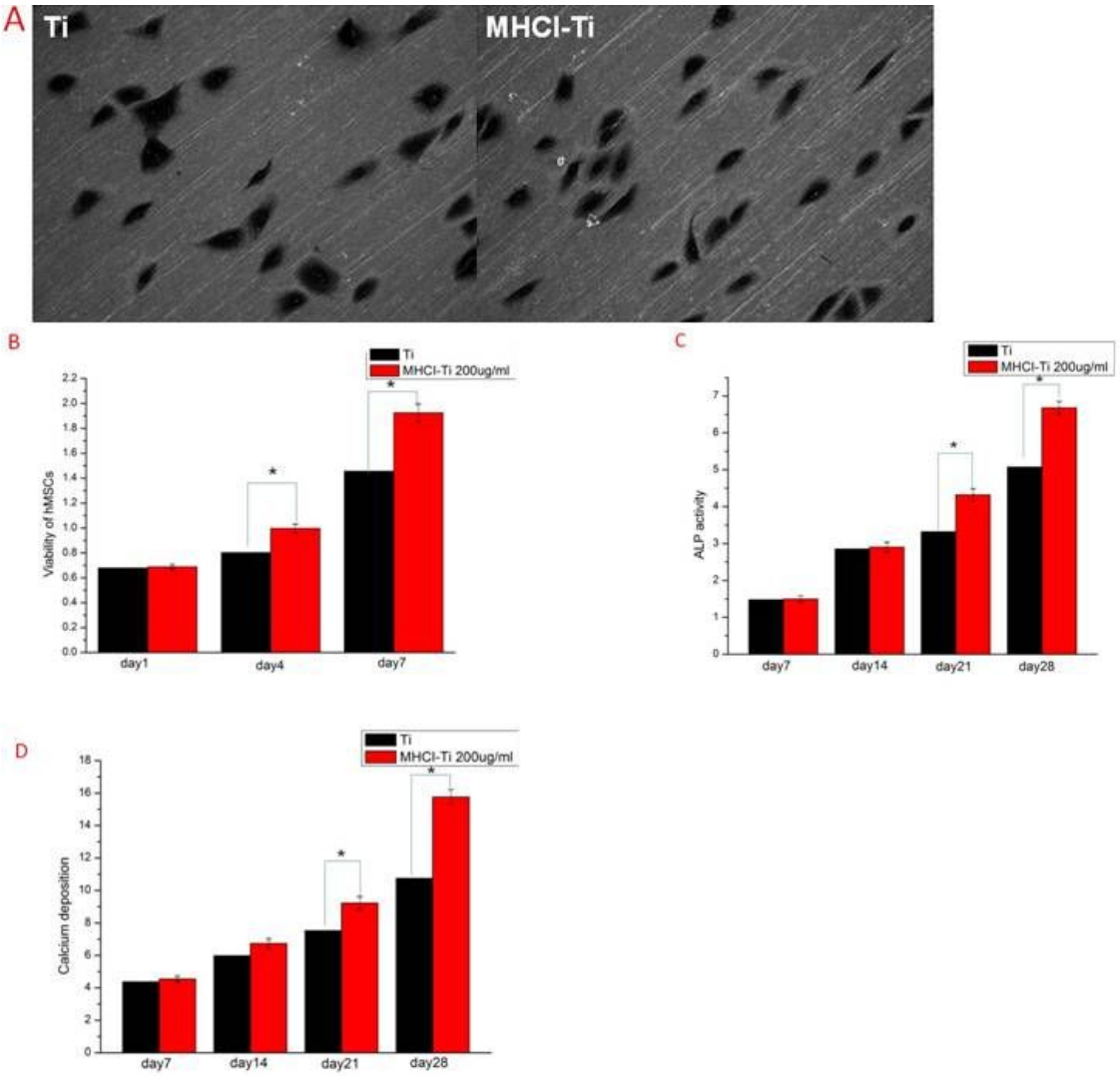

Figure 4

Osteogenic differentiation of hMSCs. (A) SEM micrographs of cells. (B) Viability of hMSCs at days 1, 4, and 7 (C) ALP activity of hMSCs over 4 weeks. (C) Calcium deposition over 4 weeks (*P < 0.01).