Materials Science and Engineering C 72 (2017) 464–471

Contents lists available at ScienceDirect

Materials Science and Engineering C

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

Alkalescent nanotube films on a titanium-based implant: A novel approach to enhance biocompatibility

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ARTICLE INFO

Article history:
Received 24 September 2016
Received in revised form 1 November 2016
Accepted 21 November 2016
Available online 28 November 2016

Keywords:
Alkalescent microenvironment
Magnesium oxide
Titania nanotubes
Biocompatibility
Titanium implant

ABSTRACT

The interfacial pH value has a marked effect on cell viability because the pro-mineralization activity of osteoblasts increases at alkaline extracellular pH, whereas the pro-resorptive activity of osteoclasts increases under more acidic conditions. To obtain the more favorable alkaline interface, we developed a novel nanotube layer that was incorporated with magnesium oxide on a titanium implant substrate (MgO/NT/Ti) via ethylenediamine tetraacetic acid (EDTA) chelation. This facile immersion-annealing process successfully created a homogeneous magnesium oxide layer with sustained release kinetics and superior hydrophilicity according to the surface characterization and microenvironment measurement. The titania nanotubes on the substrate with an anatase phase exhibited a lower passivation current and a more positive corrosion potential compared with pure titanium, which guaranteed a reasonable corrosion resistance, even when it was wrapped with a magnesium oxide layer. In vitro cell cultures showed that MgO/NT/Ti significantly increased cell proliferation and alkaline phosphatase (ALP) activity. The resulting alkalescent microenvironment created by the MgO layer encouraged the cells to spread into polygonal shapes, accelerated the differentiation stage to osteoblast and induced a higher expression of vinculin. In summary, the incorporated alkalescent microenvironment of MgO/NT/Ti provided a viable approach to stimulate cell proliferation, adhesion, and differentiation and to improve the implant osseointegration.

1. Introduction

Structural biomaterials are increasingly used in reconstructive surgery, especially for orthopedics and dental implantations such as tooth fillings and roots [1]. Upon implantation, the implant surface is surrounded by ions, proteins, sugars, and lipids that are found in tissue fluids or blood [2]. Due to the high load bearing capacity and the ability to undergo plastic deformation prior to failure, metallic materials have been frequently used as implant materials. However, most metallic implants, such as titanium, that have been widely used as dental implant materials lack the bioactivity necessary to induce osteoblast (bone) proliferation and implantation osseointegration [3]. Important surface properties, including its topography, chemistry, surface charge and hydrophilicity, can affect the interactions between implants and tissues [4]. Various surface modification methods are utilized to enhance metallic implant osseointegration.

Nanoscale morphology has been shown to be advantageous for the integration of the implant into the adjacent bone tissue because bone ingrowth is more inclined to occur in the pores [5]. It has also been reported that osteoblasts on nanophase implants exhibit increased adhesion and higher formation of calcium phosphorus minerals [6]. Therefore, titanium implants with a nanoscale surface structure are believed to be reliable and effective for enhancing the affinity between bone and the implant. Additionally, titania nanotubes (TiO_2 NTs) with open volume nanotubular structures have been shown to be ideal carriers for drug loading and release [7]. Jia et al. [8] loaded a mixture of ibuprofen/PLGA into TiO_2 NTs on titanium foil, and the synthesized drug carrier successfully demonstrated sustained drug release profiles for ibuprofen over 5 to 9 days. Wang et al. [9] attained an effective surface with both contact-killing and release-killing antimicrobial capabilities by embedding Ag nanoparticles in TiO_2 NTs and incorporating vancomycin into the nanotubes through vacuum extraction. TiO_2 NTs have great potential as a long-term drug delivery system because they provide a novel and effective strategy by adding bioactive or antibacterial agents to increase the stability of implants and raise implantation success rates.

In addition to studying the morphology, chemical composition and mechanical properties of biomaterials, surface biological modification studies have recently focused on the interaction between cells and biomaterial surfaces. It is becoming increasingly apparent that numerous biochemical aspects of the microenvironment are important regulators of cell behavior [10]. Studies have shown that osteoblast viability was markedly reduced below pH 6, and viability was significantly enhanced with an increase of pH to an optimum level at pH 8–8.5 [11–13]. The results suggested that the pH value had a noteworthy effect on cell

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http://dx.doi.org/10.1016/j.msec.2016.11.096
0928-4931/

viability and achieved its highest effect at pH 8.5. However, the importance of pH as a control factor in the biological process of implant mineralization has not been properly explored, even though it is well known that the local pH must vary correspondingly during implant degradation [12]. Therefore, we aimed to combine the nanoscale morphology as drug eluting surfaces with moderate degradation to construct an alkalescent microenvironment.

It is well-known that magnesium (Mg)-based materials have been widely applied in orthopedic surgery as degradable implants due to their resemblance to human cortical bone [14]. The degradation of Mg releases magnesium ions (Mg^{2+}), which are involved in a variety of cellular processes. Mg is the fourth most abundant cation in the human body, with approximately half of the total amount stored in bone tissue, and it serves as a co-factor for many enzymes and stabilizes the structures of DNA and RNA. In addition, Mg simultaneously increases the pH of the media [15,16]. Therefore, the resulting high concentration of Mg^{2+} without impurity ions makes magnesium oxide (MgO) a suitable source of alkalinity. In this study, we combined MgO with TiO_2 NTs using an immersion process supported by the strong chelation of ethylenediaminetetraacetic acid (EDTA) for metal ions, especially divalent metal ions such as Ca^{2+} and Mg^{2+}. In addition, the subsequent high temperature annealing helped to decompose Mg-EDTA chelates to obtain a homogeneous magnesium oxide layer with the favored anatase phase of TiO_2 NTs. Because of the simplicity of the immersion-annealing process, it could be applied to complex structures of various materials and provide significant improvements for the biological activity of implants.

2. Experimental section

2.1. Pretreatment of Ti specimens

Medically pure titanium (Ti) foil (TA 3, Baoji Titanium Industry Co. Ltd., China) was cut into discs (Φ15 mm × 1 mm), mechanically polished with 400, 800, 1200, and 2000 grit SiC emery papers and then ultrasonically cleaned in acetone, ethanol, and deionized water for 10 min, respectively.

2.2. Preparation of TiO_2 NTs array

A TiO_2 NTs array was prepared by anodic oxidation, which was conducted in a two-electrode cell as follows. The Ti discs were used as positive electrodes, and a platinum plate was used as the negative electrode. The Ti discs were anodized in a glycerol electrolyte (glycerol/water 80:20 vol.%) containing 0.13 M NH_4F at 20 V for 3 h under magnetic stirring. After anodization, the nanotube-coated Ti discs were ultrasonically cleaned in ethanol at 40 Hz for 30 s and dried in air after they were rinsed with deionized water. The pure Ti discs and the nanotube-coated Ti discs were named Ti and NT/Ti, respectively.

2.3. Loading MgO on NT/Ti

The MgO-loaded NT/Ti was synthesized using a facile immersion-annealing method and was designated as MgO/NT/Ti. For the preparation of the reaction solutions, 1.25 M ethylenediamine tetraacetic acid (EDTA) was dissolved into aqueous ammonia at room temperature, and a magnesium-rich solution was prepared by dissolving 8 M $Mg(NO_3)_2 \cdot 6H_2O$ in deionized water at 60 °C. For the deposition of MgO, the prepared nanotubes were filled with EDTA-ammonia after they were immersed in the solution, which was placed on an orbital shaker (120 r/min) at room temperature for 30 min. Subsequently, the samples were immersed in a $Mg(NO_3)_2$ solution for the chelation reactions of EDTA with the Mg ion, and this process was enhanced using the orbital shaker (120 r/min) at 60 °C for 30 min. After the samples were rinsed with deionized water to remove the excess substance covering the top of the surface, they were annealed at 500 °C for 3 h and dried in air for material characterization.

2.4. Surface characterization

A field-emission scanning electron microscope (FE-SEM, Quanta 450, FEI, USA) along with energy-dispersive X-ray spectroscopy (EDS) were used to observe the morphology and to conduct elemental analysis. An X-ray diffractometer (XRD, Ultima IV, Rigaku, Japan) was used to identify the structure of the crystalline phase in the coatings. The chemical and electronic states of the elements were determined using X-ray photoelectron spectroscopy (XPS, ESCALAB250, Thermo Fisher). Contact angle measurements were obtained using a contact angle analyzer (OCA 20, Data Physics, Germany). The average value of each substrate was analyzed by five measurements using a water drop volume of 2 μL on different spots.

2.5. Electrochemical characterization

The electrochemical properties of the samples were measured by potentiodynamic polarization using an electrochemical workstation (VersaSTAT 3, Ametek-Princeton Applied Research, USA) in a standard three-electrode system. To evaluate the stability of specimens under simulated in vivo conditions, electrochemical measurements were conducted at 37 °C in a Simulated Body Fluid (SBF) solution with similar ion concentrations to those of human blood plasma at pH 7.4, which was prepared according to a previous report by Kokubo [17]. The working electrode was each test specimen, whereas a platinum plate and a saturated calomel electrode (SCE) were employed as the counter and reference electrodes, respectively. Polarization curves were measured after 1 h of immersion in the solution to obtain a relatively stable state. The scan was applied from −1.5 V vs.SCE to +1.5 V vs.SCE with a scan rate at 1 mV s^{-1}.

2.6. Microenvironment measurements

An immersion test was performed to monitor the Mg^{2+} released from the MgO/NT/Ti samples. Thymol blue was used as an acid-base indicator to directly manifest the pH range of the surface, and the color transitioned from red to yellow at pH 1.2–2.8 and from yellow to blue at pH 8.0–9.6. MgO/NT/Ti was soaked in 15 mL of phosphate buffered saline (PBS) for varying periods (1, 3, 5, 7 and 14 days) to examine Mg^{2+} release. Three parallel samples were studied, and the solution was collected and replaced with 15 mL of fresh PBS at each time interval. The Mg^{2+} concentration was measured using an atomic absorption spectrophotometer (AAS, MKIIM6, Thermo, USA).

2.7. Cell culture and statistical analysis

MC3T3-E1 cells were cultured on MgO/NT/Ti, NT/Ti and Ti to evaluate the biocompatibility and bioactivity. The cells were cultured on the specimens in a 24-well plate with an initial cell density of 3×10^4 cells/well.

Cell morphology and adhesion were imaged with a fluorescence microscope-confocal laser scanning microscope (CLSM, TCS SP8, Leica, Germany). Cells cultured for 3 days were stained. The cells were fixed and permeabilized using 4% paraformaldehyde and 0.5% Triton X-100 in PBS. After the cells were washed in PBS and incubated with 5% normal non-immunological goat serum, they were subjected to mouse monoclonal anti-vinculin at 4 °C overnight, followed by 1 h of further incubation with secondary antibody fluorescein isothiocyanate (FITC)-conjugated phalloidin and tetramethylrhodamine (TRITC)-conjugated phalloidin at room temperature. Finally, the cells were incubated with 4′,6-diamidino-2-phenylindole (DAPI) for 3 min at room temperature and were then washed three times with PBS.