Materials Science and Engineering C 78 (2017) 96–104

Contents lists available at ScienceDirect

Materials Science and Engineering C

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

Enhanced osteogenic activity of anatase TiO_2 film: Surface hydroxyl groups induce conformational changes in fibronectin

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ARTICLE INFO

Article history:
Received 19 December 2016
Accepted 10 April 2017
Available online 11 April 2017

Keywords:
Atomic layer deposition
Titanium dioxide
Cell-binding site
Osteoblast compatibility

ABSTRACT

In this study, with an attempt to identify the effects of TiO_2 crystalline phase compositions on the osteogenic properties, the anatase and rutile TiO_2 thin films with similar film thickness, surface topography and hydrophilicity were prepared on Si (100) substrates by atomic layer deposition (ALD), subsequent thermal annealing and ultraviolet irradiation. The films were studied with XRD, XPS, FE-SEM, AFM, FTIR and contact angle measurements. In vitro cellular assays showed that the anatase phase led to better osteoblast compatibility in terms of adhesion, proliferation, differentiation, mineralization as well as osteogenesis-related gene expression when compared with the rutile phase. We investigated the difference between the anatase and rutile TiO_2 films at the biomolecular level to explain the enhanced osteogenic activity of the anatase film. It was found that the presence of more Ti—OH groups on anatase surface induced more cell-binding sites of fibronectin (FN) exposed on its surface, causing a more active conformation of the adsorbed FN for subsequent osteoblast behaviors.

1. Introduction

Titanium and its alloys have been widely used in dental and orthopedic implants due to their excellent mechanical properties and biocompatibility [1,2]. The biocompatibility of titanium is believed to be closely related to the existence of native titanium oxide (TiO_2) thin layer on its surface [3]. Recently, the TiO_2 crystalline phase composition (anatase and rutile) [4] has attracted great attention because it has been found to play an important role in osteogenic properties of TiO_2.

Previous studies have reported the effect of anatase and rutile TiO_2 on osteoblast behaviors, yet conflicting results were reported. He et al. fabricated TiO_2 films using reactive DC magnetron sputtering technique, and found that the number of osteoblasts and the level of alkaline phosphatase (ALP) activity on anatase were significantly higher than those on rutile. Considering the similar surface topography of both the films, the authors attributed the enhanced cellular behavior of the anatase TiO_2 film to its better wettability [5]. Li et al. reported the augment of rutile content up-regulated the ALP activity of human osteosarcoma cells cultured on the micro-arc oxidation treated Ti surface due to the increase of surface roughness [1]. An et al. synthesized anatase, rutile and the mixture of them through heat treatment at different temperatures. Mouse osteoblasts showed higher levels of cell activity on the mixture of anatase and rutile than on the single phase. After the investigation on the relative influence of various factors, they concluded that the higher surface roughness of the mixed structure contributed to the better osteogenic property [6]. Since surface topography, hydrophilicity and crystalline phase compositions can all affect cellular behaviors, thus the previous studies of the influence of anatase and rutile TiO_2 on osteogenic properties reported contradictory results [1,5]. In this scenario, in order to study the effect of phase compositions of TiO_2 on osteoblast behaviors, it is necessary to minimize the influence of surface roughness and hydrophilicity.

Atomic layer deposition (ALD) is a gas-phase thin film deposition method with the advantages of good adhesion of films on substrates, excellent step-coverage and pinhole-free deposition [7,8]. The ALD method can be used to deposit TiO_2 thin films with similar surface topography, thus minimizing the influence of surface roughness. Additionally, the ALD method allows relatively low growth temperatures [9], which makes it easier to obtain the metastable anatase phase. With proper heat treatment, anatase phase can be totally transformed into rutile phase, as indicated by previous studies [10]. The difference between anatase and rutile thin films on the hydrophilic ability can be minimized through proper ultraviolet (UV) treatment without influencing their crystal structure and surface topography [11,12].

In this paper, we aimed to study the effect of TiO_2 crystalline phase compositions on the osteogenic properties and explore the influence mechanism. Anatase and rutile TiO_2 thin films with similar surface roughness were successfully prepared by ALD technology and subsequent post-deposition annealing. The film thickness and hydrophilicity were also kept similar between these two films. The amount and cell-binding sites of the adsorbed proteins on the anatase and rutile thin

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http://dx.doi.org/10.1016/j.msec.2017.04.056
0928-4931/

films were investigated. The osteogenic behaviors of MC3T3-E1 pre-osteoblasts on the anatase and rutile thin films were examined. The mechanism of the enhanced osteogenic activity of anatase TiO_2 film was also discussed.

2. Materials and methods

2.1. Preparation and characterization of the anatase and rutile TiO_2 thin films

TiO_2 thin films were grown on 1 cm × 1 cm Si (100) wafers in an ALD reactor (TFS-500, Beneq, Finland). Titanium tetrachloride ($TiCl_4$) and deionized water were used as the precursors. Ar of 99.999% purity was used as the purge gas. Each ALD cycle used for TiO_2 film growth consisted of the following four steps in sequence: (1) 500 ms exposure to $TiCl_4$, (2) 5 s Ar purge, (3) 500 ms exposure to H_2O, and (4) 5 s Ar purge. During deposition the gas pressure in reactor chamber was maintained around 8 mbar. The deposition temperature was 200 °C. The number of ALD cycles was kept at 2000.

The as-deposited TiO_2 thin film was pure anatase phase. Thermal annealing experiment was performed for the as-deposited anatase films at 1000 °C in the atmosphere for 1 h to obtain the pure rutile phase. In order to achieve similar hydrophilicity between anatase and rutile films, UV irradiation was carried out with a 15 W black light bulb (wavelength 254–365 nm, ZF-90, China). Because the photo-induced hydrophilic surface of TiO_2 gradually returns to the initial state in several days [12], the cellular assays were performed immediately after UV irradiation.

The crystal structure of the TiO_2 films was analyzed by X-ray diffraction (XRD, RigakuD/Max-2200 PC, Japan) with CuK_α radiation at 40 kV and 40 mA. The elemental composition of the obtained films was confirmed by X-ray photoelectron spectroscopy (XPS, ESCALAB 250, Thermo Scientific, USA). The surface and cross-section morphologies of the TiO_2 films were observed using a field emission scanning electron microscope (FE-SEM, Hitachi S-4800, Japan). The surface groups on the films were analyzed by a Fourier transform infrared spectroscopy (FTIR, NICOLET iS50, Thermo Scientific, USA) using the reflection mode. The thickness of the TiO_2 films was measured by a spectroscopic ellipsometer (SE, VB-400, J.A. Woollam Co., USA). The roughness analysis was performed using an atomic force microscope (AFM, Bruker Dimension Icon) in ScanAsyst mode. The water contact angles were measured with a contact angle meter (SL200B, KINO Industry Co., Ltd., USA).

2.2. Quantitation of protein adsorption and exposed cell-binding sites

In order to investigate the conformation of adsorbed protein, human plasma fibronectin (FN) was chosen as a model protein in this study. Adsorption of FN (Sigma, U.S.A.) was performed by immersing samples in 20 μg/ml FN solution at 37 °C for 2 h. A microBCA Protein Assay Kit (Beyotime Biotechnology, China) was used to analyze the total amount of adsorbed fibronectin. The exposed cell-binding sites were examined by enzyme-linked immunosorbent assay (ELISA) and immunofluorescence staining using monoclonal antibody HFN7.1. The FN-adsorbed samples were washed in PBS and blocked in 1% BSA at room temperature for 1 h, and then incubated with HFN7.1 (DSHB, U.S.A.) at 37 °C overnight. After rinsed in PBS with 0.1% Tween 20, all samples were incubated with a secondary antibody (HRP-conjugated goat anti-mouse IgG, CWBiotech, China) at ambient temperature for 1 h, and subsequently immersed in 3,3′,5,5′-tetramethylbenzidine (TMB, Beyotime Biotechnology, China) for 20 min in darkness. After that, the reaction supernatants were

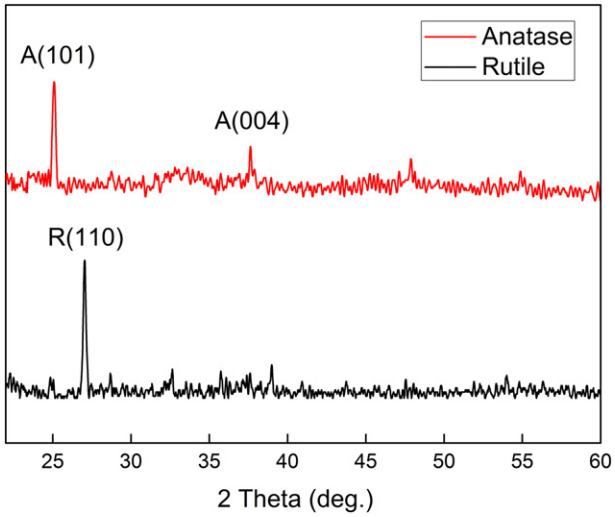

Fig. 1. X-ray diffraction patterns of the anatase and rutile TiO_2 thin films.

Table 1
Primers for real-time polymerase chain reaction (PCR).

Target gene	Direction	5′–3′ primer sequence
Runx2	F	5′-GCACCTACCAGCCTCACCATAC-3′
	R	5′-ACAGCGACTTCATTCGACTTCC-3′
OPN	F	5′-CTTTCACTCCAATCGTCCCTAC-3′
	R	5′-CCTTAGACTCACCGCTCTTCAT-3′
OCN	F	5′-CAGAGTCCAGCAAAGGTGCAGC-3′
	R	5′-TCAGCCAACTCGTCACAGTCCG-3′
BSP	F	5’-TCCTCCTCTGAAACGGTTTCC-3′
	R	5′-GGAACTATCGCCGTCTCCATT-3′

F, forward; R, reverse.

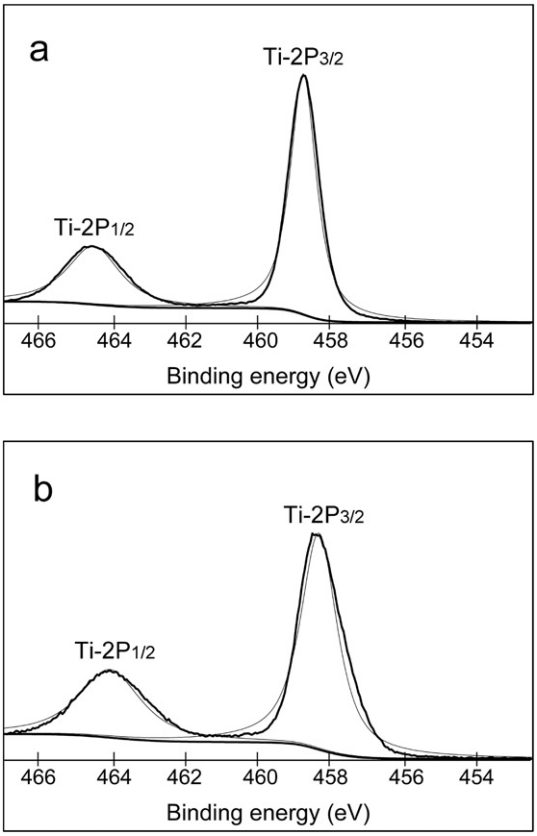

Fig. 2. XPS spectra of (a) anatase and (b) rutile TiO_2 thin films.