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Original article

Proliferation and differentiation of osteoblastic cells on silicon-doped TiO_2 film deposited by cathodic arc

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ABSTRACT

This study aimed at the proliferation and differentiation of osteoblastic cells on silicon-doped TiO_2 and pure TiO_2 films prepared by cathodic arc deposition. The films were examined by X-ray photo-electron spectroscopy, which showed that silicon was successfully doped into the Si-TiO_2 film. Meanwhile, no significant difference was found between the surface morphology of silicon-doped TiO_2 and pure TiO_2 films. When osteoblastic cells were cultured on silicon-doped TiO_2 film, accelerated cell proliferation was observed. Furthermore, cell differentiation was evaluated using alkaline phosphatase (ALP), type I collagen (COL I) and osteocalcin (OC) as differentiation markers. It was found that ALP activity, the expression levels of OC gene, COL I gene and protein were up-regulated on silicon-doped TiO_2 film at 3 and 5 days of culture. Moreover, no significant difference was found in apoptosis between the cells cultured on silicon-doped TiO_2 and pure TiO_2 films. Therefore, findings from this study indicate that silicon-doped film favors osteoblastic proliferation and differentiation, and has the potential for surface modification of implants in the future.

1. Introduction

Titanium (Ti) and Ti alloys have been widely used in orthopaedic and dental implants because of their good mechanical properties. However, Ti and its alloys are bioinert materials that are unable to integrate chemically with bone and cannot accelerate the healing of bone tissues at the early stage after implantation [1]. These implants might be encapsulated by fibrous tissues, which are deemed as an important reason that leads to mechanical loosening and implant failure [2]. It is a good solution to improve physiochemical osseo-integration by surface modification of implants, which can improve bone-implant contact [3]. Depositing a bioactive film on implants is a prospective method to improve integration of Ti-based implants to surrounding bones because the bioactive film can produce lasting physiochemical osseo-integration, reduce implant loosening and ameliorate other adverse reactions [4].

In order to reach rapid osseo-integration, Ti implants are normally coated with hydroxyapatite (HA) using plasma-spraying technique. HA coating has been shown to improve osseo-integration between the implant and bone, and has already been used in clinical application [5]. However, low bonding strength and rapid dissolution of HA coatings could lead to degradation, peeling, and fatigue-induced failure under tensile loading [6], which has limited the scope of its biomedical applications. Various deposition techniques and other bioactive materials have been investigated to obtain a stronger bond between the implant and bioactive materials. Coating prosthesis with TiO_2 has been studied and the TiO_2 thin films have displayed better mechanical properties and stronger bond than HA [7,8]. Several deposition methods including physical vapor deposition and chemical vapor deposition have been developed to deposit TiO_2 thin films over the last decade [9,10]. Among the physical vapor deposition techniques, cathodic arc deposition is an effective technique. Materials can be kept at low temperatures during treatment while the films could still grow dense with a fine microstructure on their surfaces, leading to improve mechanical properties and corrosion resistance. Cathodic arc deposition is advantageous to produce thin films due to its high deposition rate and adherence of the films [11,12]. The close adherence would avoid an interphase among bone, bioactive film and implant metallic substrate, supporting favorable conditions for long-term anchorage of implanted device.

Silicon (Si) has been found to be an essential trace element for the growth and development of normal bones and cartilages [13]. Carlisle [14] and Schwarz and Milne [15] first reported that silicon

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deficiency in chicks and rats would lead to abnormality of bones and defective cartilaginous tissues, both of which could be restored upon the addition of soluble Si to their diet. This experiment indicated that Si may play an important role in bone and cartilage tissue metabolism. A clinical report [16] showed that the increase of dietary Si intake in men and premenopausal women could lead to a higher bone mineral density. In addition, Si-containing glasses or ceramics has been proved to promote osteoblast proliferation, differentiation and collagen production [17,18]. Therefore, doping Si into films of artificial implants is an attractive idea for enhancing biocompatibility and bioactivity of implant, reducing early implant loosening and prolonging the lifespan of artificial implants. Implant coated with doping Si ceramics showed improved stability, providing a stronger interface between the coating and the implant and enhancing bone growth and mineralization [19,20]. However, Si-doped TiO_2 film deposited by cathodic arc deposition has not been thoroughly studied and the effects of biological responses of the film are not clear by now.

Therefore, this study will focus on modification of titanium surface by depositing Si-doped TiO_2 film via cathodic arc deposition technique. The biological effects of Si-doped TiO_2 film were evaluated by culturing MG63 cells, which have been frequently used in vitro testing of biomaterials [3,21].

2. Materials and methods

2.1. Preparation of films on titanium substrates

Two types of commercial pure titanium plates were prepared with the diameters of 5.8 mm and 31 mm, and a thickness of 1 mm. The plates were polished mechanically, degreased by sonication in acetone, ethanol and deionizer water in sequence and dried in air condition. Then, the samples were processed with deposition using filtered Ti and Si cathodic arc plasma sources codeposition on titanium plates in oxygen atmosphere, which were labeled as Si-TiO_2. For comparison, the samples codeposition using double Ti cathodes were labeled as TiO_2. In codeposition process, there was a direct current superimposed pulsed bias with 15% duty ratio applied. The direct current voltage was 40 V and the bias was −500 V. The pulse duration of cathodic current was 2000 μs and the pulse frequency was 7 Hz. The deposition time was 1 h and the working pressure was 4.0×10^{-3} Pa.

2.2. Surface characterizations

Surface morphologies of the films were observed using scanning electron microscopy (SEM, XL-30, Philips, Holland). X-ray photoelectron spectroscopy (XPS, MicroLab 310-F, USA) was employed to analyze the elements of the films. The contact angles between the water drops and samples were measured by sessile drop method [22]. The contact angle was determined by an Automatic Contact Angle Meter (SL200B, Solon, China). The reported results were the averages of three independent measurements.

2.3. Cell culture

Human osteoblast-like cell line MG63 cells (Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, China) were used to evaluate proliferation and differentiation behavior on the TiO_2 and Si-TiO_2 film surfaces. After ultrasonic cleaning in ethanol and sterilization by autoclaving, the plates were put into culture wells and cells were seeded onto these plates at an initial density of 1×10^4 cells/cm^2 in triplicate. MG63 cells were cultured with 10 mM β-glycerophosphate (Sigma, G9891), 50 μg/ml ascorbic acid (Sigma, A4403) and 10% fetal bovine serum (FBS) in a Dulbecco's modified Eagle's medium (DMEM) at 37 °C in a humidified atmosphere with 5% CO_2. The culture medium was renewed every two days.

2.4. Cell morphology

The 5.8 mm diameter of commercial pure titanium plates deposited with TiO_2 and Si-TiO_2 films were prepared. MG63 cells were seeded at a concentration of 1×10^4 cells/cm^2 on the TiO_2 and Si-TiO_2 film surfaces respectively in 96-well plates. For SEM observation, the cells on the plates were cultured for day 1 and 5. Three samples were randomly collected from each group, washed with PBS, and fixed with 2.5% glutaraldehyde buffered by PBS. The samples were then dehydrated in graded alcohols (50%, 70%, 90% and 100%), critical point dried, and sputter-coated with gold in sequence. The morphology of the cells was observed using the SEM.

2.5. Cytotoxicity test

To investigate whether the Si-TiO_2 film affected cell apoptosis induced by the doping of Si, flow cytometric spectra was employed. MG63 cells were seeded at 1×10^4 cells/cm^2 on the 5.8 mm diameter of TiO_2 and Si-TiO_2 film surfaces, and cultured in 96-well plates for day 1 and 5. The viability and apoptosis of the cells was determined using the Cell Apoptosis Kit with Annexin V Alexa Fluor 488 & PI, V13241 (Invitrogen, USA), and was visualized by flow cytometry using an Epics XL flow cytometer (Beckman Coulter). This method is based on the binding properties of Annexin V to phosphatidylserine (PS) and the DNA-intercalating capability of propidium iodide (PI).

2.6. Cell proliferation assay

The 5.8 mm diameter of titanium plates deposited with TiO_2 and Si-TiO_2 films were prepared. MG63 cells were seeded at a concentration of 1×10^4 cells/cm^2 on the TiO_2 and Si-TiO_2 film surfaces respectively in 96-well plates. Cells grew on the plates for day 1, 3 and 5. Cell proliferation was analyzed using Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Tokyo, Japan) with measuring dehydrogenases' metabolic activity through a tetrazolium reaction. In the presence of dehydrogenases, the tetrazolium reaction produced formazan, a yellow and water-soluble produce. The amount of the formazan was proportional to the number of living cells that could be measured the absorbance at 450 nm by a thermo multiscan EX plate reader (Thermo Multiskan MK3 plat reader, USA).

2.7. ALP activity

ALP activity of the cells growing on the 5.8 mm diameter of TiO_2 and Si-TiO_2 film surfaces was used as the key differentiation marker for assessing the expression of the osteoblast phenotype. ALP activity was measured quantitatively through p-nitrophenol formed from the enzymatic hydrolysis of p-nitrophenylphosphate (pNPP). Cells were seeded at 1×10^4 cells/cm^2 and cultured for day 1, 3 and 5, and then washed twice with PBS. 120 μl of the pNPP solution (Wako Pure Chemical, Japan) was then added to the washed cells. After the cells was incubated for 1 h at 37 °C, 120 μl of 1.0 M NaOH solution was added to stop the activities of the phosphatase enzyme and to convert p-nitrophenol (colorless) to p-nitrophenolate (yellow). The quantity of p-nitrophenol was estimated by measuring the absorbance at 492 nm due to p-nitrophenolate. The ALP activity was normalized with the culture area to compare the dependence of osteogenic activity.