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Improved bone-forming functionality on diameter-controlled TiO_2 nanotube surface

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Abstract

The titanium dioxide (TiO_2) nanotube surface enables significantly accelerated osteoblast adhesion and exhibits strong bonding with bone. We prepared various sizes (30–100 nm diameter) of titanium dioxide (TiO_2) nanotubes on titanium substrates by anodization and investigated the osteoblast cellular behavior in response to these different nanotube sizes. The unique and striking result of this study is that a change in osteoblast behavior is obtained in a relatively narrow range of nanotube dimensions, with small diameter (~30 nm) nanotubes promoting the highest degree of osteoblast adhesion, while larger diameter (70–100 nm) nanotubes elicit a lower population of cells with extremely elongated cellular morphology and much higher alkaline phosphatase levels. Increased elongation of nuclei was also observed with larger diameter nanotubes. By controlling the nanotopography, large diameter nanotubes, in the ~100 nm regime, induced extremely elongated cellular shapes, with an aspect ratio of 11:1, which resulted in substantially enhanced up-regulation of alkaline phosphatase activity, suggesting greater bone-forming ability than nanotubes with smaller diameters. Such nanotube structures, already being a strongly osseointegrating implant material, offer encouraging implications for the development and optimization of novel orthopedics-related treatments with precise control toward desired cell and bone growth behavior.

Keywords: TiO_2 nanotubes; Osteoblast; Cell adhesion; Cell elongation; Alkaline phosphatase activity

1. Introduction

It is well known that titanium (Ti), with a thin, conformal, titanium oxide (TiO_2) surface, is a biocompatible orthopedic material which provides an excellent physical bonding with the surface of bone. The bone bonding generally occurs without the common connective tissue layer that forms from the body's immune response (foreign body reaction) between the implant metal and the underlying bone surface [1–3]. While a thin TiO_2 passivation layer on the Ti surface can impart improved bioactivity and better chemical bonding to the bone [4], other techniques have been developed to further enhance the bioactivity of a pure Ti surface, such as direct coating of bioactive materials like hydroxyapatite and calcium phosphate [5–7]. However, even though these surface-modified layers have good bioactivity and a high surface area, they tend to delaminate at the interface between the implant and the bone due to the relatively large, micrometer-regime thickness of the coated layer on Ti [8], presumably due to the stress accumulation commonly seen in a thick coating of foreign material. In order to overcome this problem, a number of plasma-sprayed Ca–Si-based ceramic coatings [9,10] have been developed, but their roughness and layer thickness are still in the micrometer range. For the purposes of this study, we focus on nanoscale thickness surface coatings. It is therefore desirable to

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develop an implant with a bioactive surface layer that has a high surface area for enhanced bonding yet is thin enough – in the nanometer range – to minimize delamination.

Our recent reports indicate that modifying Ti surfaces with TiO_2 nanotubes for orthopedic applications significantly enhances mineral formation [11], the adhesion of osteoblasts in vitro [12] and strongly adherent bone growth in vivo [13], showing better bone bonding characteristics than conventional micro-roughened Ti surfaces by sandblasting. Human mesenchymal stem cells (hMSCs) have also been shown to preferentially differentiate into osteoblast-like cells on the TiO_2 nanotube surface [14]. A physical advantage of the TiO_2 nanotube surface system is that it is composed of and created directly from the underlying native Ti constituent, unlike the foreign ceramic and spray coatings on Ti or the Ti alloyed surfaces mentioned previously. In addition, the nanotube layer is at most ~300 nm thick, which is much thinner than the previous coatings, and this nanometer length scale eliminates the tendency of delamination prevalent in thicker, micrometer-scale layers.

Nanostructures have recently been of great interest due to their high surface-to-volume ratio and the greater degree of biological plasticity compared to microstructures. In terms of biomaterial development and implant technology, the cellular response can be affected by topographical circumstances. In the field of in vitro cell biology, there is a growing body of data that shows how cells respond positively to nanotopography [9,10,12–21]. It has been proven that cells sense and react to nanotopography, in vitro as well as in vivo, by exhibiting changes in cell morphology, orientation, cytoskeletal organization, proliferation, signaling and gene expression [9,10,12–21].

The interface reaction between a Ti implant and newly grown bone plays an important role in preparing orthopedic implantation materials with ideal properties [5]. It is critical to evaluate the surface structure and topography effects for optimal bone growth and implant success. The fabrication of nanostructured titanium dioxide (TiO_2) nanotube arrays has been a primary subject of investigation lately due to the wide range of TiO_2 nanotube applications in the fields of photocatalysis [22,23], photoelectrolysis [24], sensing [25], solar cell [26] and biomaterials [12–15,20,21,27]. We have demonstrated that the presence of vertically aligned TiO_2 nanotube surface structuring on Ti foils in biological applications had a critical effect that improved the proliferation and mineralization of osteoblasts [12], enhanced the mobility, vasodilating bioactivity and endothelialization of endothelial cells [14] and up-regulated osseointegration into rabbit tibia in vivo [13], and preferentially induced hMSC differentiation into osteoblasts [15] because of the unique nanotopographical features and high-quality biocompatibility of the TiO_2 nanotube surface. We have also observed a rather significantly enhanced adhesion of myocytes on such nanotube surfaces (data not shown).

The purpose of this work is to investigate the in vitro behavior of osteoblasts cultured on vertically aligned TiO_2 nanotubes with various inner pore (30–100 nm) diameters, and to investigate the nanosize effect on osteoblast cell adhesion, morphology and osteogenic functionality for biomaterial implant optimization. Here we report a unique variation in cell behavior even within such a narrow range of nanotube dimension.

2. Materials and methods

2.1. TiO_2 nanotube fabrication

TiO_2 nanotube surfaces were created using a two-electrode-setup anodization process, as described previously [11,12]. Briefly, a 0.25 mm thick cp-Ti sheet (Alfa-Aesar, 99.5% metals basis, USA) was used for this process. The nanotubes were prepared in a 1:7 volumetric ratio of acetic acid ($\geqslant$99.99% purity, Sigma–Aldrich) to 0.5% w/v hydrofluoric acid in water (48% w/v, EM Science, USA) at 5, 10, 15 and 20 V for 30 min. A platinum electrode (99.9% pure, Alfa-Aesar, USA) served as the cathode. The samples were then washed with deionized water, dried at 80 °C and heat-treated at 500 °C for 2 h in order to crystallize the as-fabricated amorphous structured TiO_2 nanotubes into an anatase structure. The anatase phase was confirmed by Raman spectrometry using an argon laser (Horiba model iHR320 imaging spectrometer, at 514.5 nm wavelength). The samples ($1.27 \times 1.27\ cm^2$) used for all experiments were sterilized by autoclaving prior to use. A flat Ti sheet cut into identically sized pieces was used as a control after being chemically cleaned by acetone and isopropanol for 10 min in an ultrasonic cleaner, dried and autoclaved.

2.2. Atomic force microscopy

An atomic force microscope (AFM) was used to characterize the roughness of the samples. The AFM apparatus was a Veeco scanning probe multi-mode microscope with a nanoscope IV controller. The average roughness (R_a) was measured for all experimental samples (Ti and 30–100 nm TiO_2 nanotube surfaces) in tapping mode using Micromasch tapping cantilever tips (NSC15/NoAl) over a 1.0 μm^2 scan area.

2.3. Contact angle measurement

The measurement of contact angle for the 30–100 nm TiO_2 nanotube surfaces was carried out by a video contact angle measurement system (Model No. VSA 2500 XE, AST Products, Inc.).

2.4. Osteoblast cell culture

For these studies, MC3T3-E1 mouse osteoblast (CRL-2593, subclone 4, ATCC, USA) were used. Each 1 ml of cells was mixed with 10 ml of alpha minimum essential

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