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Full Length Article

Interaction of osteoblast -TiO_2 nanotubes *in vitro*: The combinatorial effect of surface topography and other physico-chemical factors governs the cell fate

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ABSTRACT

In the context of surface modification of implants by TiO_2 nanotubes, the topography of the nanotubes has been considered as a sole modulator of cellular response of adherent cells. However, a recent report has indicated about the existence of other contributing factors. Keeping this contradiction in mind, here we have performed a comprehensive study to account the contribution of the various factors such as morphological aspects, physico chemical aspects and the topographical features of TiO_2 nanotubes on the cellular response. Here, the TiO_2 nanotubes of varied topography were synthesized on the Ti6Al4V surface through anodic oxidation. SEM analysis showed that there was a variation in tube diameter, cross-sectional length, tube density and wall thickness along with the variation in oxidation time and voltage. An attempt to co-relate the cellular response of osteoblast cells (MG-63) cultured on the nanotubes has revealed that except cell proliferation; cell adhesion, spreading, vinculin distribution (focal adhesion) and differentiation (ALP activity) do not possess any direct relationship with the nanotube topography. Further, by comprehending the cellular responses with the variation in the extent of anatase phase (XRD study), surface roughness and wettability of nanotube surfaces, we established that the variation in cellular responses is a combinatorial effect of surface topography and other physico-chemical properties of TiO_2 nanotubes. This study will help in developing bioactive implant surfaces modified with TiO_2 nanotubes, for clinical application.

1. Introduction

Titanium and its alloys have been widely used in orthopaedic implants because of their superior mechanical properties, low elastic modulus, excellent corrosion resistance and good biocompatibility. However, they fail to bond directly to bone tissue *in vivo* because of their bio-inert nature. In the past decade, a number research groups have focused on the surface modification of Titanium based implants to improve the bioactivity of the implant surface and its subsequent osteointegration. Recently, it has been observed that the nanostructured TiO_2 especially nanotubular TiO_2 is remarkably more bioactive in comparison to the bulk Titanium [1]. TiO_2 is a stable oxide of Titanium and often found as a native layer, that forms spontaneously when Titanium is exposed to ambient air at room temperature [2]. The passive TiO_2 layer itself imparts biocompatibility and corrosion resistance to the Titanium surface. There is a rising amount of data pertaining to the improved biological performance of Titanium implants having surfaces modified with TiO_2 nanotubes [3–7]. So far, significant attempts have been made to optimize the morphology of the TiO_2 nanotubes to improve the biological function of the material [8,9]. Although these reports individually highlighted the influence of a single process parameter on nanotube morphology, yet there is a dearth of a comprehensive report describing the combined effect of oxidation time and applied voltage on nanotube morphology.

It has long been established that formation of TiO_2 nanotubes enhances the adhesion of cells, in comparison to a bare Ti surface. Das et al. showed the preferential attachment of OPC1 human osteoblast on nanotube surface compared to that on bare Ti surface [10]. It is well documented that morphological attributes of the TiO_2 nanotubes such as tube diameter and tube wall thickness have a vital influence on the formation of focal adhesion points which in turn strongly affects the cell adhesion and proliferation. Different research groups have also reported the improvement of initial cell adhesion on small diameter nanotubes ($\leq$30 nm), using various cell types [3,11,12]. Interestingly, this firmly established

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view point has been contradicted in a recent report published in 2015. The authors suggested that nanotopography impaired the formation of focal contacts and FAK expression and proposed that this may be due to the reduction of total contact surface available. Nonetheless, the high proliferation of cells was observed at an early stage and it was attributed to the existence of some other potential compensation signalling pathways [13].

A critical analysis revealed that the available literature majorly focused on the impact of nanotube diameters on cell response, however, the combined influence of TiO_2 nanotube surface topography related features such as surface roughness, wettability, crystallinity of the oxide layer, protein adsorption has not been considered at the time of analysis. These surface characteristics often determine the process of osteo-integration of an implant by influencing a number of cellular events such as cell adhesion, migration proliferation, differentiation and apoptosis [14–17].

This set of literature clearly suggests that a comprehensive analysis is essential to decipher the relationship between the process parameters, TiO_2 nanotube morphology, surface characteristics of nanoscale TiO_2 layers and cellular responses. Keeping these perspectives in mind here we have conducted a detailed study on anodic oxidation based surface modification of Ti6Al4V to find out (i) the correlation between process parameters (oxidation time and voltage) and the morphological attributes and surface characteristics of TiO_2 nanotube formed, and (ii) to decipher the combined influence of nanotube structure (tube diameter and wall thickness) and bulk properties of the surface modified with TiO_2 nanotubes layer on bone cell compatibility. Anodic oxidation was chosen as a method of surface modification as it can produce highly organized nanostructures in a small time frame. Here, we have oxidized the Ti6Al4V surfaces by varying the oxidation time and applied voltage. The phase analysis of the oxide layer was carried out by XRD. The morphology of the nanotubes (tube diameter, wall thickness, and distribution) was inspected by SEM and further analysed using image analyser. Surface characterization was performed by a surface profiler and contact angle measurement. We further studied the protein adsorption behaviour and biomineralization *in-vitro*. Cellular responses were investigated using bone cell line (MG-63). For this purpose, both quantitative and qualitative cell adhesion studies were performed by Trypan blue assay and SEM, focal adhesions were studied by immunostaining of vinculin. Further, proliferation of MG63 cells was reported by MTT assay and the differentiation status of the cells was investigated using Alkaline Phosphatase assay (ALP).

2. Materials and methods

2.1. Ti6Al4V specimen preparation

Medical grade Ti6Al4V cold-rolled sheet with 0.90 mm thickness was procured (Classic Alloys, India). It was sectioned into rectangular blocks and polished using Silicon Carbide grit sheets (1/0, 2/0, 3/0, 4/0) to remove stains, scratches and the native oxide layer. Thereafter, the samples were cloth polished, using alumina suspension. All samples were degreased and cleaned in ethanol for 30 min, in an ultrasonic bath (APL digital).

2.2. Fabrication of TiO_2 nanotubes

Anodic oxidation of the polished samples was carried out by using a conventional two-electrode configuration with a DC power supply (APLAB). Ti6Al4V was used as an anode and a graphite rod as a cathode. The electrodes were kept apart in the electrolyte at a distance of 2 cm throughout the study. The electrolyte was composed of 0.25% ammonium fluoride in 90% vol. ethylene glycol and 10% vol. distilled water, as demonstrated by reference [4]. The fabrication of TiO_2 nanotube was carried out with variation in oxidation time and voltage around 30 V and 3 h respectively. The selected process parameters taken for studies are given in Table 1 along with the respective group names. After performing anodic oxidation, the sample was detached from the anode and washed in ethanol in an ultrasonic bath for 8 min. Further, all oxidized samples were heat treated in a muffle furnace at 500 °C for 3 h.

2.3. Phase analysis and morphological characterization

X-Ray Diffraction technique (Rigaku, D/MAXULTIMA) was used to confirm the formation of oxide layer after anodic oxidation and heat treatment. The surface morphology of anodized samples was inspected by FESEM (Nova Nano Sem450, FEI).

2.4. Analysis of surface roughness and topography

The surface roughness of both polished and oxidized samples was measured using a surface profiler (VeecoDektak 150). The instrument generates data based on a single linear traverse of a mechanical stylus, over the sample surface. Profiling was performed at three locations spanning the entire oxidized region of the Ti6Al4V samples. A length of 1000 μm was scanned for 80 s in a single trace. Ra (Average Roughness) value was calculated by considering the mean of average roughness values obtained after three traces. Further, the topography of the polished substrate and TiO_2 nanotubes grown under three different voltage conditions were characterized using an atomic force microscope (AFM), VEECO diInnova, operating in contact mode, with a 0.01–0.025-Ω cm antimony-(n)-doped Si tip (Bruker, model RESPA-20). The average roughness (Ra) was determined using a scanning area of 3×3 μm with a scan rate of 0.5 Hz. Further, 3D image analysis and roughness calculation was performed using WSxM 5.0 Develop 9.0 software.

2.5. Wettability study

To evaluate the wettability of oxidized surfaces, Contact angle meter (Data Physics OCA20) was used. For the assessment of surface hydrophilicity and hydrophobicity, the contact angle was measured with respect to ultrapure water, at three different locations on each sample. The contact angle for each drop was recorded by calculating the mean of left and right angles. The average of three readings has been reported. The polished specimen was used as a control.

2.6. Protein adsorption assay

The protein adsorption behaviour of the nanotubular TiO_2 surface in comparison to the polished Ti6Al4V specimen was evaluated using Bradford assay. All the samples used in the study were of equal size (0.5×1 cm^2). Bovine serum albumin (BSA) and Lysozyme were used as model proteins for the study. The assay was performed using a method outlined elsewhere, with modifications [18]. Briefly, samples were placed in 24 well polystyrene plate and 1 ml aqueous protein solution (1 mg/ml) was added to each well. Protein adsorption was carried out at 37 °C for 24 h. Subsequently, the supernatant was collected and the absorbance of residual protein was determined using a spectrophotometer (Systronix, double beam spectrophotometer 2203) at 595 nm, using Bradford assay. The corresponding protein concentration was evaluated using standard graphs of BSA and Lysozyme respectively. The amount of protein adsorbed to the sample surface was calculated by deducting the residual protein concentration from the initial protein concentration.

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