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Electrochemical anodic oxidation process of porous titanium granules for biomedical applications

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Abstract. Titanium granules can be used as bone graft substitute in the field of orthopedic and periodontal surgery, but their bone bonding ability needs to be promoted. Nanostructured materials have shown to enhance bioactivity and the overall bioperformance of biomaterials. In the present study, the effects of time and voltage of anodic oxidation process are investigated in order to form TiO_2 nanostructures with optimized morphology on the surface of porous titanium granules. The anodized granules are subsequently heat treated at 450°C for 1 h. MG63 osteoblast-like cell is used to evaluate cell attachment and viability on the surfaces of anodized and annealed granules using Scanning Electron Microscopy (SEM) and dimethylthiazol-diphenyl tetrazolium bromide (MTT) assays, respectively. The results of anodizing process show that TiO_2 nanostructures are constructed at the voltage of 60 V for 3 h. The X-ray diffraction results show improved crystallinity of TiO_2 nanostructures on the surface of annealed anodized granule surfaces after the annealing process. Cell culture experiments show improved cell spreading and viability on the surface of annealed anodized granules compared to those on the anodized sample. It is concluded that annealed anodized granules have the potential for orthopaedic and periodontal applications as bone graft substitutes.

1. Introduction

Titanium (Ti) and titanium alloys are the materials of choice for most dental and orthopedic applications [1]. The thin TiO_2 passivation layer that usually forms on titanium surface in oxidative media is considered to be responsible for titanium-based materials biocompatibility [2,3]. However the bone bonding ability and direct contact of titanium-based implants with the bone tissue have been challenges for biomaterials researchers [2].

It is known that the surface of biomaterials firstly encounters with the biological milieu, consisting of biomacromolecules and cells. As a result, the surface characteristics will directly affect the cellular responses in the early phase of implantation [4]. Various methods for surface modification have been utilized to improve the osseointegration of an orthopaedic biomaterial [5]. The topographical cues at the nanoscale level may provide enhanced bone-biomaterial interactions and improve the integration of bone and materials [6]. Hence, nanoscale surface modification of an implant surface could be considered as an option that contributes to the mimicry of local cellular environments and favors the process of bone formation for improved osseointegration [7].

Titanium oxide nanostructures are prepared by

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various techniques, such as sol-gel, electrophoretic deposition, and anodization. The anodization process is usually preferred to as the sol-gel or electrophoretic methods as it provides strongly adherent TiO_2 layer on the surface of titanium-based materials [3]. The formation of titania nanostructure layer on the surface of titanium substrates via anodization in fluorine solution is widely studied since it is a simple, straightforward, cost effective, controllable, and reproducible method. It is shown that this method creates a self-organized and highly ordered titanium dioxide layer [8-10]. It is believed that the nanostructured TiO_2 thin films obtained by this method are usually amorphous [11]. However, it is reported that cells are attached on the crystalline TiO_2 surfaces more readily as compared to the surfaces with lower crystallinity. Since the crystalline structures can also provide a better osteointegration [12,13], heat treatment is employed to enhance surface crystallinity of metallic substrates, and therefore their biocompatibility.

The effect of anodizing parameters on the formed TiO_2 characteristics and morphology has been studied by many groups. Si et al. and Hamlekhan et al. studied the effects of anodic voltages on the morphology and wettability of porous titanium dioxide structures, respectively [8,14]. Narayanan et al. investigated the effect of electrolyte parameters on the surface roughness of titanium dioxide structures [3]. Although the anodization of titanium sheets or implants has been previously reported, there is no study on the anodization of irregular shaped materials, such as titanium granules.

The objective of the present study is to optimize the effect of anodization parameters such as applied voltage and oxidation time on the formation of TiO_2 nanostructures on the surface of porous titanium granules. In order to improve the crystallinity of TiO_2 nanostructures on the surface of anodized titanium granules, heat treatment is employed after the anodizing process. Morphology of the formed TiO_2 nanostructures is studied by Scanning Electron Microscopy (SEM). X-Ray Diffraction (XRD) is used to determine the crystal structures of the samples. In order to find out the biological responses to the treated granules, morphology and viability of osteoblast-like cells MG-63 (human osteoblast-like) are also investigated by SEM and MTT assays, respectively.

2. Materials and methods

2.1. Electrochemical oxidation of titanium granules

The titanium granules used for anodization process (042459, Alfa-Aesar) were cleaned by immersion in acetone and ethanol, rinsed with deionized water (DI water), and dried prior to anodization. The anodization process was carried out at controlled temperature between 4-10°C in a two-electrode cell with titanium granule as the anode and stainless steel plate as the cathode. The electrolyte was a mixture of ethylene glycol (1.00949, Merck, 99%) and 0.38 wt% ammonium fluoride (NH_4F) (1.01164, Merck) dissolved in 2 vol% DI water. The electrochemical oxidation process was performed at voltages of 30, 60, 65, 70, and 80 V for 1.5 and 3 h (Table 1). The anodized granules were subsequently annealed for 1 h at 450°C to obtain crystalline structures. The samples used for this study are listed in Table 2.

Table 1. The voltage and time used in the anodizing process.

Voltage (v)	Time (h)
30	1.5
30	3
60	1.5
60	3
65	3
70	3
80	3

Table 2. The samples and their abbreviations.

Porous titanium granule	G
Anodized titanium granule	AG
Annealed anodized titanium granule	AAG

2.2. Characterization of the samples

The G, AG, and AAG samples were gold-sputtered and their surface morphology was characterized by SEM (S160, Cambridge, UK) at an accelerating voltage of 15 kV in high vacuum mode. The phase composition of the samples was also evaluated by X-Ray Diffraction (XRD) (X'Pert Pro MPD, PANalytical) using a Cu target ($K_\alpha, \lambda = 1.54056$ Å).

2.3. Cell culture

Human osteoblast-like cell (MG63) provided by American Type Culture Collection (Cellular bank of Pastor Institute, Iran) was used for this study. The cells were cultured in T25 plastic flasks (Nunc) in alpha minimum essential medium (α-MEM, Invitrogen, Corporation, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The growth medium was changed every 48 h. Cultured cells were detached by trypsinization, suspended in fresh culture medium, and used for the experiments. Commercial titanium granules (Tigran Technologies AB, Sweden) were used as control for each set of experiments.

2.4. Cell viability

The cell viability of the samples was evaluated through colorimetric MTT assay (Roche Diagnostics GmbH, Germany) in 24 well culture plates. Cells were seeded on the granules with a density of 10^4 cell/ml and incubated for 3 days. After the incubation time, culture medium was removed and 0.1 ml DMEM with 0.01 ml MTT solution was added to each well. After 4 h incubation, the crystallized formazan dye was solubilized by adding 0.1 ml of 10% sodiumdodecyl sulfate (SDS) in 0.01 M HCL to each well. The absorbance was read at 580 nm using an ELISA plate reader (Labsystems multiscan, Netherlands).

2.5. Cell adhesion and morphology

Morphological characteristics of cells on the surface of samples were investigated with a SEM. Cells grown on the samples for 3 days were first washed with PBS, fixed with 2.5% glutaraldehyde (Sigma Aldrich), and dehydrated in graded series of ethanol-water baths (20, 30, 50, 60, 70, 80, 90, 95, and 100%, v/v). The surface of the samples was sputter coated with a 15 nm layer of Pt/Pd and examined in SEM.

3. Results and discussion

3.1. Anodic oxidation process

The SEM results in Figure 1 present the morphology of G sample (Figure 1(a)) and the samples which are anodized at different voltages for different oxidation times (Figure 1(b)-(h)). The surface structure of the G sample in Figure 1(a) shows porous structure with micro pores. At the lowest applied voltage, 30 V, with duration of 1.5 h (Figure 1(b)), no significant morphological changes were observed, while by increasing voltage to 60 V, the porous TiO_2 structure was produced on the surface of sample (Figure 1(c)). At the voltage of 30 V, with duration of 3 h, a compact TiO_2 layer was formed (Figure 1(d)). When this process was performed at voltages of 60 V, it led to the production of TiO_2 nanostructure, which is shown in Figure 1(e). The anodizing at higher voltages, i.e. 65 V and 70 V, resulted in destruction of the TiO_2 layer (Figure 1(f) and (g)). At the highest applied voltage, 80 V, the morphology of the surface changed dramatically to the extent that the titanium granules were damaged significantly (Figure 1(h)).

The formation of dense oxide layer can be explained using the following equations:

$$2H_2O \rightarrow O_2 + 4H^+ + 4e, \quad (1)$$

$$Ti + O_2 \rightarrow TiO_2. \quad (2)$$

The mechanism of pore formation by field-assisted chemical dissolution of dense oxide via interfering F^- ion is presented in the following equation:

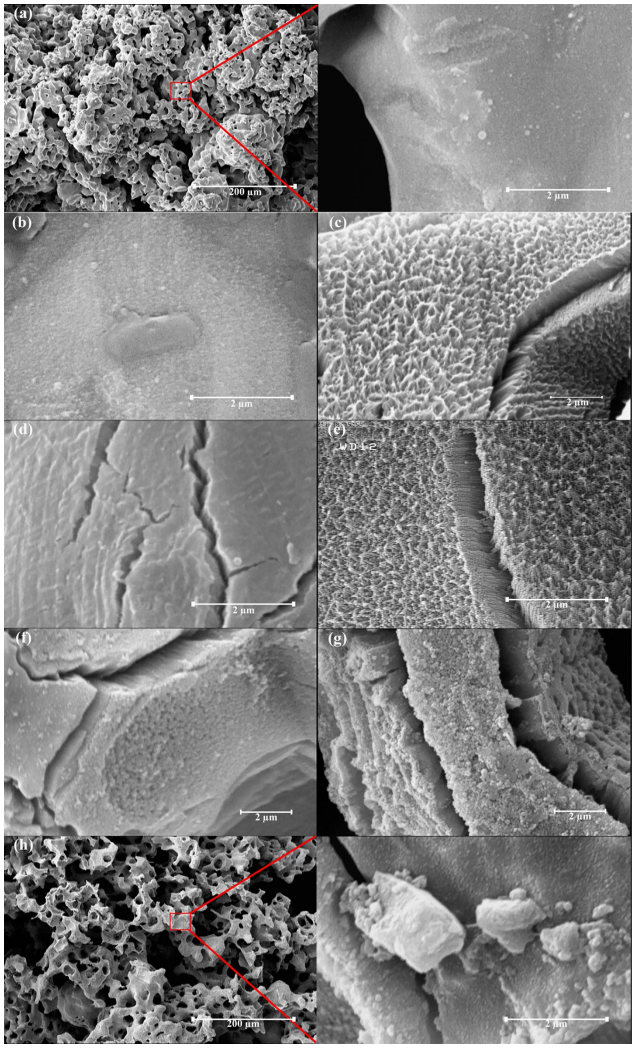

Figure 1. SEM images of a) G samples; AG sample anodized at: b) 30 V for 1.5 h, c) 60 V for 1.5 h, d) 30 V for 3 h, e) 60 V for 3 h, f) 65 V for 3 h, g) 70 V for 3 h, and h) 80 V for 3 h.

$$TiO_2 + 6F^- + 4H^+ \rightarrow [TiF6]^{2-} + 2H_2O. \quad (3)$$

Based on this equation, it is apparent that the oxide layer is attacked in fluoride media under the formation of highly soluble $[TiF6]^{2-}$ complex and a porous TiO_2 layer is obtained [15-17].

In the present study, anodizing needed a potential higher than 30 V in order to induce migration of F^- ion toward the anode electrode. The higher voltages at 60 V for 1.5 h could cause penetration of fluoride ions into the oxide layer and induce dissolution of the TiO_2 layer. The longer periods of anodizing process around 3 h create deep pore structures leading to porous TiO_2 nanostructures as detected.

During anodization, the newly-formed oxide layer on the anode is a dielectric barrier to the current flow and it keeps growing until reaching the dielectric breakdown limit. Generally, the anodized layer is not

uniform due to the existence of flaws, defects, local stress, and non-uniform oxide thickness, which could be found in granules with irregular shapes. When the applied voltage increases, the potential drop at the weak points exceeds the dielectric limit so that sparking happens. The local temperature at these points can be up to several thousand Kelvin and lead to a local melting process. Thermal stressing of these anodized titanium leads to the multiplication of weak points, and, consequently, breakdown of the dielectric [18]. The voltages higher than 65 V can result in destruction of the TiO_2 layer. Disintegration of the granules and TiO_2 nano structures may cause insufficient strength of the granules leading delamination and separation of the TiO_2 layer.

It is believed that anodizing at 60 V for 3 h is the best condition for producing the TiO_2 nano structures on the surface of the irregular shaped Ti granules. By applying 60 V potential for 3 h oxidation, we can benefit TiO_2 nanostructure surface which is created directly from the underlying titanium substrate. This can eliminate the tendency of delamination that occurred prevalently in the bioactive coating [19].

3.2. Heat treatment of the samples

XRD spectra of G, AG, and AAG samples are shown in Figure 2. In XRD patterns of G and AG samples (Figure 2; lower and middle patterns), the associated peaks of Ti could be observed at 2θ of 35.474, 38.722, 40.503, 53.285, 63.238, 70.886, 74.448, 76.438, and 77.591. In AAG spectrum (Figure 2; upper pattern), the major peaks of anatase (JCPDS Nos. 21-1272) in 2θ of 25.316 and rutile (JCPDS No. 21-1276) in 2θ of 27.412 are appeared. This could explain the amorphous structure of oxide layer formed in AG sample after the anodizing process. The heat treatment after anodizing process resulted in the formation of crystalline anatase and rutile phases.

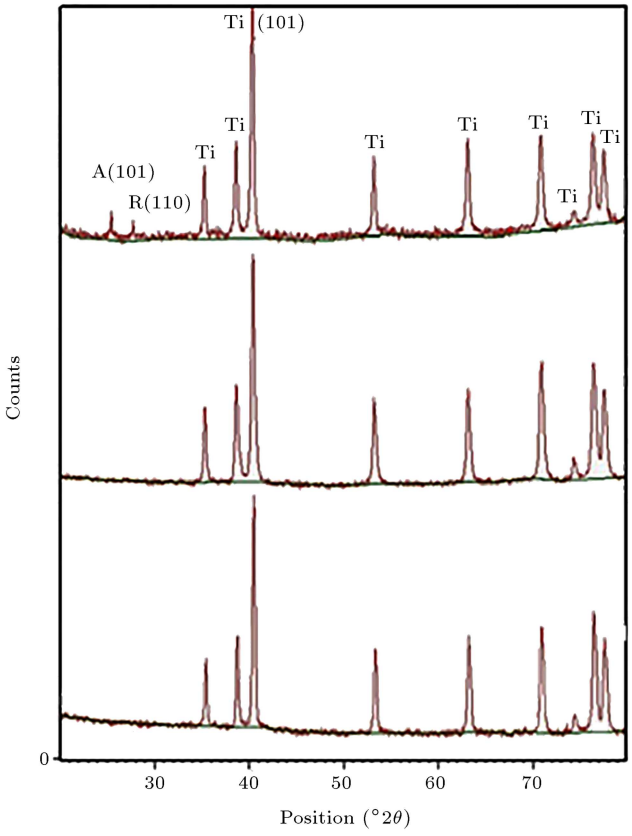

Figure 2. XRD spectra of G (lower pattern), AG (middle pattern), and AAG samples (upper pattern).

3.3. Cell morphology

MG63 cell morphology was investigated after 3 days of culture (Figure 3). Cells were spread out on the surface of all substrates as shown with the circles in Figure 3(a)-(c). There were no significant morpho-

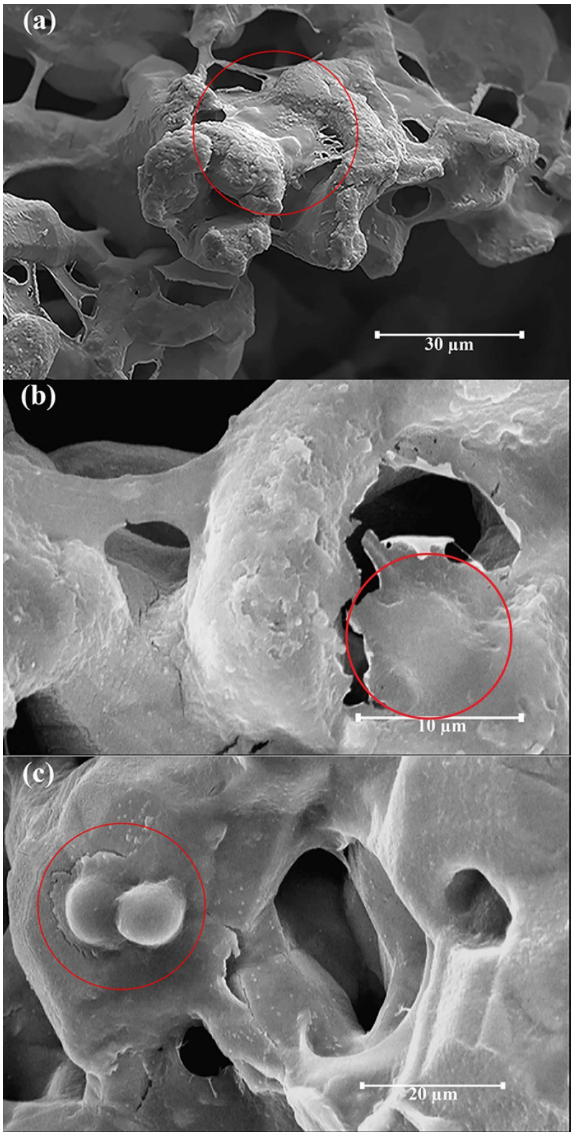

Figure 3. SEM images of MG63 cells cultured on the a) G sample, b) AG sample, and c) AAG sample.

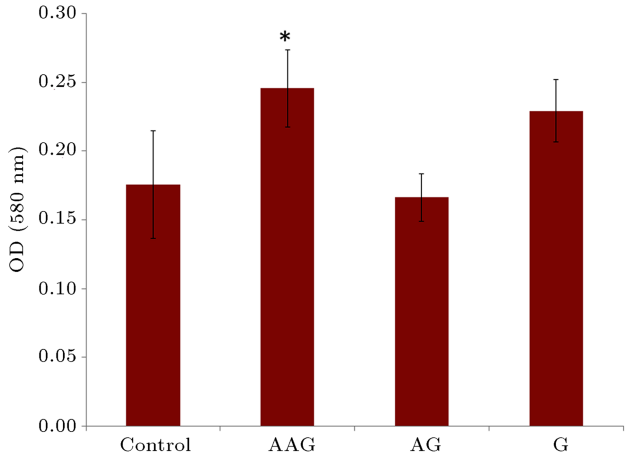

Figure 4. MG63 cells viability on control, AAG, AG and G samples. $*p < 0.05$ compared to other samples and control.

logical differences between MG63 cells in the three types of samples. The results of cell morphology are in agreement with previous research by Chang and Wang [20] as well as Bai et al. [21] that revealed similar cell morphologies on anodized and annealed anodized substrates.

3.4. MTT assay

The viability of MG63 cells was tested using a commercially available MTT assay kit. In MTT assay, spectrophotometric measurement of the net absorbance was calculated to determine the concentration of viable cells. All the samples showed no cytotoxicity as they revealed the similar or higher absorbance values compared to the control. The cell viability on AAG samples was higher than those on both AG and G samples (Figure 4).

Crystallinity is one of the factors that affects cellular behaviour and response [22]. It is shown that cell attachment and viability is high on the combination of anatase and rutile as compared to amorphous TiO_2 structure [19]. Attachment and growth of the osteoblast cells enhanced for anatase-rutile TiO_2 nanostructure of AAG sample compared to G sample. This could be due to the formation of anatase and rutile phases and probably higher hydrophilic surface of AAG sample [21,23].

Anodic oxidation has shown to enhance the surface biocompatibility of metallic substrates [24], whereas, here, anodizing process showed the adverse effect on cell viability which is possibly correlated with the residual fluorine from NH_4F remained within the pores of the non-annealed amorphous nanostructures introduced during the anodization process. The anion uptake is quite common for oxide layers grown by electrochemical modifications, which can be annihilated to large extent by annealing due to evaporation of HF and F_2 species [21,25]. This has been reported, previously, by Bai et al. [21] as they produced titania nano tubes by applying fluorine for anodizing titanium foil. Also, Barbier et al. believed that the fluoride must be actively considered as a potent toxic compound [26]. Even though the toxicity of fluoride is largely neglected in some applications, like dental toothpastes, it is considered mainly toxic in cellular systems, even at low doses [26].

4. Conclusions

The present study aims to optimize the effect of anodization parameters, such as applied voltage and oxidation time, on the formation of TiO_2 nanostructures on the surface of porous titanium granules for bone tissue engineering applications. The results show that anodizing process at 60 V for 3 h creates TiO_2 nanostructures which could be transformed to anatase and rutile phases with subsequent annealing process. The lower anodizing voltages and times (30 V for 1.5 h and 3 h as well as 60 V for 1.5 h) do not efficiently form nanostructures. The anodizing process at 65, 70, and 80 V for 3 h caused destruction of TiO_2 nanostructure; also, the morphology and structure of porous titanium granules drastically changed during the application of 80 V. The anodized annealed granules showed improved cell attachment and growth with no cytotoxic effects. It can be concluded that the annealed anodized porous titanium granules would have a great potential for orthopaedic and periodontal applications as filler and bone graft substitutes.

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Biographies

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