HOS cell adhesion on TiO$_2$ nanotubes texturized by laser engraving

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Abstract. Due to its outstanding properties, the titanium and its alloys have been widely used in the dental and orthopaedic fields as biomaterials. The TiO$_2$ nanotubes surface and the texturized process by laser engraving enables significantly accelerated osteoblast adhesion on the biomaterial. For this reason in this paper, the HOS cell responses on TiO$_2$ nanotubes fabricated on Ti6Al4V alloy and texturized by laser engraving were evaluated. The test surfaces were carried out on smooth Ti6Al4V as control, TiO$_2$ nanotubes (NT) and surfaces with micropoints obtained by laser engraving, with 1mm spacing (NTP1) and 0.5mm (NTP2). The results show that the texturized process enables decreases the contact angle thus improving wettability of the TiO$_2$ nanotubes surface. The NTP1 and NTP2 surfaces show excellent cell adhesion and spreading on the surface, which is evident in epifluorescence microscopy images. Furthermore, the NTP1 and NTP2 surfaces improved the cell proliferation at 18% and 16% respectively in relation with NT surface, showing that the laser texturing improves cell response of TiO$_2$ nanotubes.

1. Introduction

The biomaterials were developed in order to fulfill needs that could not be met naturally [1], for this reason this materials are used in a large number of applications, bone implants, joints, vascular stents, skin substitutes, plastic surgery, cranial and dental implants among many others [2-4]. Bioactive materials used in orthopaedic implants to improve the integration of the implant to the bone and enhanced osseointegration process [5, 6]. One of the most widely used materials for this purpose is titanium and its alloys, Ti6Al4V alloy mainly because of their excellent properties.

Currently different methods are being studied to improve the surface properties of titanium and its alloys in order to achieve and accelerate the osseointegration process in the surface of these materials. One of the most widely used methods has been the electrochemical anodization process [7-9]. This method allows grow controlled the titanium oxide layer, which is formed naturally in the surface of this material. Modifying anodizing parameters, it can be generated different morphologic surfaces and properties that can improve the material behaviour to be implanted in the human body. Another method for modifying the surfaces of orthopaedic implant materials is the textured by laser engraving [10-14]. This method has improved the cell adhesion of Ti and Ti6Al4V surfaces by creating patterns on surfaces. These patterns become places where a preferential cell adhesion occurs, which increase the adhesion of osteoblast cells.

Therefore, in this work we study the effect of laser-textured surface in the cellular response of TiO2 nanotubes grown on Ti6Al4V.
2. Experimental

2.1. Sample preparation

Ti6Al4V cylindrical disc with 14mm diameter and 3mm thick was grinding with silicon carbide paper in successive grades from 220 to 1200 grit and subsequently the disc were cleaned by sonication in ethanol and deionized water for 15 minutes. Some these substrates were anodized to get TiO2 nanotubes, theses substrates were immersed in a dilute acid mixture of nitric acid (HNO3) and hydrofluoric acid (HF) for 1 minute in order to remove the thin oxide layer that spontaneously forms on the Ti6Al4V surface in presence of the air. The anodization process was carried out in a conventional two electrodes cell, using a solution of 0.5wt% HF, applying 10V during 1 hour at room temperature. Fourth different surfaces were used in this study. They include untreated surface (Ti6Al4V), TiO2 nanotubes (NT TiO2), and laser engraving with two different circular patterns (NT TiO2 P1 and NT TiO2 P2). The patterning surfaces were obtained by laser engraving using a methodology previously described in [15] using a sealed CO2 laser with maximum power of 40W. The circular patterns generated on the surface can be seen in the Figure 1. All samples were sterilized in autoclaved at 120°C for 20 minutes and then by UV radiation for 30 minutes on each side.

2.2. Biological test

These test were performed using a methodology previously described in [15]. For this, HOS cells was cultured in 25cm² Falcon culture plates and maintained in an incubator at a temperature of 37°C regulated with 5% CO2, 95% air, and saturated humidity. RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin was used as the cell-cultured medium. For adhesion assay, 2.5×10⁴ cells/ml in cultured medium were placed on each material and incubated at 37°C. After 1, 3 and 5 days the cells were detached using 0.5ml Trypsin-EDTA for 5 minutes and counted microscopically in a Neubauer chamber. In addition a fluorescence staining were made directly in each sample using Hoechst 33342 fluorescent stain and epifluorescence microscopy (Nikon Eclipse E4000), B-2A filter (Ex=450-490, DM=500, Ba=515). Images were obtained using a camera Nikon Coolpix 5000. UV2A. Each time experiments were performed in duplicate and the results were calculated as number of cells per surface area.

3. Results and discussion

The TiO2 nanotubes morphology grown on Ti6Al4V alloy by applying 10V in bath containing 0.5% HF concentration is shown in Figure 2. The nanotube inner diameter in the mouth is approximately 30nm. The formation of nanotubes preferentially grown in the alfa phase along with beta phase,
besides dissolution of the oxides was observed. Such morphology has been widely described for TiO$_2$ nanotubes grown in acid electrolytes with F containing [16,17].

![Figure 2](image)

**Figure 2.** Ti6Al4V surface morphology (a) microstructure with nanotubular layer (b) TiO$_2$ nanotubes morphology surface.

As shown in the Figure 3, the patterns has a semicircular shape with diameter of 150µm, the separation spots on the vertical axes is about 1250µm and the horizontal axes is 1295µm for NT TiO$_2$ P1 and 630µm for NT TiO$_2$ P2 respectively. This variation in the dimensions of the patterns was previously described in [15] and the movement performed by the laser engraving equipment to achieve surface texturing the surface attribute.

![Figure 3](image)

**Figure 3.** Patterns on TiO$_2$ nanotubes morphology (a) pattern 1 and (b) pattern 2.

The hydrophilic/hydrophobic behaviours of the surfaces are shown in the Figure 4. Ti6Al4V smooth surface have a hydrophilic behaviour while the anodized surface had a hydrophobic behaviour. This hydrophobic behaviour has been widely reported [18,19]. The TiO$_2$ nanotube surfaces texturized by laser engraving (NT TiO$_2$ P1 and NT TiO$_2$ P2) show a reduction in a contact angle, making these anodized surfaces less hydrophobic. Previously it reported that the laser texturing process on smooth
surfaces of Ti6Al4V generates an increase in hydrophobicity [15], which it is contradictory to what is observes in this study.

Figure 4. Contact angle.

Ti6Al4V, TiO2 nanotubes and TiO2 nanotubes texturized by laser engraving result non-toxic to HOS cells, this results consistent with previously reported in the literature [20-22]. Moreover, as shown in Figure 5, the HOS cell adhesion show a high affinity to surfaces with TiO2 nanotubes. The cell adhesion process is favoured on these surfaces due to the roughness and increased surface area generated by these nanometrics structures [20, 22]. The Figure 6 shows a representative images of the HOS cells during three incubation times, as can be seen the HOS cells were randomly oriented and well dispersed. On the TiO2 nanotube surface at 5 culture days the HOS cells days is possible appreciate.

Figure 5. Cell adhesion on the surfaces.
4. Conclusions
This study produced nanotube arrays with a uniform tube diameter through the anodization of Ti6Al4V. These surfaces were texturized by laser engraving. In cell culture experiments, the nanotube and nanotube texturized surface exhibited adhesion and spreading that was more rapid than that of untreated surface, despite a higher contact angle. These results provide evidence that nanotube texturized can significantly enhance cell activity. It was shown that the texturized by laser engraving is an effective means of promoting cell functions on nanostructured surface implants, thereby improving the bone-implant interface in vivo.

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References
[1] M R Souza et al 2016 Mater Res Express 3 035401
[2] C H Ku et al 2002 Biomaterials 23 1447
[3] A A John et al 2015 RSC Adv 5 39232
[4] E Beltran Partida et al 2015 Materials 8(3) 867
[5] C Yao et al 2007 J Biomed Mater res 85(1) 157
[6] P Sevilla et al 2010 J. Phys.: Conf. Ser. 252 012009
[7] P Schumki et al 2011 Angew. Chem., Int. Ed 50 2904
[8] M Kulkani et al 2015 Nanotechnology 26 062002
[9] Menglin Li et al 2013 Nanotechnology 24 305706
[10] M Bereznai et al 2003 Biomaterials 24 4197
[11] Milan Trica et al 2006 Applied surface science 253 2551
[12] A Y Vorobuev 2007 Applied Surface Science 253 7272
[13] Khosrroshahi M E et al 2007 Applied Surface Science 253 8772
[14] N Mirhosseini et al 2007 Applied surface science 253 7738
[15] A Sandoval Amador et al 2016 J. Phys.: Conf. Ser. 687 012012
[16] Buyng Gwan L 2009 Trans Nonferrois Met Soc China 19 842
[17] M Rossi de Souza 2014 Revista Materia 19 53
[18] E Balaur et al 2005 Electrochem. Commun 7 1066
[19] J L Rosa et al 2014 Arch Helath Invest 3(5) 43
[20] A S Zuruzi et al 2006 Nanotechnology 17 531
[21] S Bauer et al 2008 Acta biomaterialia 4 1576
[22] K S Brammer et al 2009 Acta Biomaterialia 5 3215