TiO₂ Nanostructures: Voltage Influence in Corrosion Resistance and Human Osteosarcoma HOS Cell Responses

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Abstract

Objectives: To develop TiO_2 nanostructures using an electrochemical process and evaluate the influence of voltage in the generation of nanotubes and the adhesion of human osteosarcoma cells on anodizing Ti6Al4V surfaces. **Methods/ Statistical Analysis:** TiO_2 nanostructures on Ti6Al4V in a solution of 1M H₃PO₄ + 0.2% wt HF for 1 hour at 14 V, 20 V and 25 V were obtained. Surface morphology was evaluated by using scanning electron microscopy and the corrosion behavior of the anodized surfaces was studied using potentiodynamic polarization and Electrochemical Impedance Spectroscopy (EIS). Cell toxicity was evaluated using a colorimetric MTT assay and the cell morphology was reviewed using a fluorescence microscope. **Findings:** TiO_2 nanotubes with diameters of 54.35 nm, 90.84 nm and 85.02 nm were obtained by anodizing at 14 V, 20 V and 25 V respectively. Using an anodizing process an organized and uniform structure was obtained with a density of 130/µm², 60/µm² and 6/µm², for the samples anodized at 14 V, 20 V and 25 V respectively. The anodized samples presented nanotubes with intertubular spaces between 10 and 14 nm. The results showed a lower corrosion rate of the anodized surfaces compared to the base material (UT-Ti64), in addition, it was observed that the samples with higher cell count adhered to its surface have higher cell viability percentages, 80% for the samples anodized at 20 V and 25 V. **Application/Improvements:** The results show that nanostructures could be customized depending on the applications such as higher corrosion resistance and better transport of nutrients favoring the cell metabolism.

Keywords: Cell Adhesion, Corrosion Resistance, Nanostructures, TiO,

1. Introduction

Titanium and its alloys are biocompatible materials that have different properties such as excellent corrosion resistance, good strength to density ratio, fatigue resistance and formability which are necessary in the use of biomedical applications^{1,2}. However, titanium alloys may present toxicity problems related to the presence of alloying elements, which is the case of the Ti6Al4V alloy³, where elements such as aluminum and vanadium increase the chances of generating toxicity in the implant. These elements, upon release, can cause local tissue irritation, inflammation reactions and other possible complications leading to failure and early withdrawal of the implant⁴.

In order to improve the performance of titanium alloys, different surfaces modification techniques such as

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PVD, thermal oxidation, sol gel, laser treatments, cathodic spray, anodic oxidation and others have been applied^{5,6}. Within these techniques, the anodic oxidation process allow the generation of a barrier or tubular type film that allows a better integration in the implant-bone relationship, being a great alternative for this type of materials⁷. The electrolytes that favor the growth of barrier-like layers are mainly electrolytes of dilute solutions of sulfuric, phosphoric and acetic acid, among others^{8,9}, where their main advantage lies in improving the adhesion and bonding of the formed oxide layer with the substrate¹⁰. For the growth of tubular layer, aqueous or organic electrolytes with fluoride ion additions are used. Within the aqueous electrolytes are the dilutions of HF and an electrolyte composed of NH₄H₂PO₄ and NH₄F¹¹. The most used organic electrolytes are composed of different glycerol or ethylene glycol ratios with small additions of water and NH₄F. TiO₂ nanotubes evince an increasing interest due to their photoelectron chemical, photocatalytic and bioactive properties and their respective applications in dye-sensitized solar cells, gas sensors and biomedical implants¹²⁻¹⁴.

The aim of this work was to study the effect of the voltage on the behavior of the TiO_2 nanostructures grown on the Ti6Al4V alloy, their morphology, composition and their influence on corrosion behavior and cell adhesion.

2. Materials and Methods

2.1 Materials and Sample Preparation

Ti6Al4V ELI alloy (ASTM F136) discs with 12.7 mm diameter and thickness of 3 mm were used in this project. The Ti6Al4V ELI alloy samples were ground with silicon carbide emery papers up to 1200 grit size and polished with diamond pastes of 3 and 1 μ m average particle size. After polished the samples were ultrasonically cleaned with ethanol for 10 minutes and air-dried at room temperature.

2.2 Anodizing

The anodizing process was used to modify the surface of the Ti6Al4V samples, we used a two electrodes electrolytic cell, and the Ti6Al4V ELI disc was used as anode and a stainless-steel plate as cathode. The electrolytic solution of 1 M H_3PO_4 0.2% v/v HF was used to obtain nanotube morphology. The applied voltages were 14, 20 and 25 V. In all cases, the anodizing time was 3600 seconds. After

the anodization process the samples were rinsed with distilled water and air dried at room temperature.

2.3 Surface Characterization

The morphology of the nanotubes was analyzed by a Scanning Electron Microscopy (SEM), using a scanning electron microscope model Quanta FEG 650 equipped with X-ray dispersive spectroscopy EDX (Apollo X).

2.4 Electrochemical Measurements

The equipment used for electrochemical measurement consisted of a three electrodes cell, a computer controlled potentiostat/galvanostato Gamry 600, a graphite high purity rod was used as a counter electrode and Ag/AgCl (3M KCl) as reference electrode. Ringer's solution (8,4 g/L NaCl, 0,302 g/L KCl, 0,298 g/L CaCl₂.2H₂O, 0,15 g/L NaHCO₃) was used as electrolyte. The electrochemical impedance spectrum measurements were acquired in a range from 105 Hz to 10-2 Hz applying signal amplitude of 10 mV. The potentiodynamic polarization behavior of the samples was performed applying a scan from -500 mV to +2500 mV (versus open circuit potential) at a scanning rate of 1 mV/s. All the electrochemical experiments were carried out at 37°C.

2.5 Biological Test

Prior to the cell culture experiments, samples of Ti6Al4V and TiO, nanotubes were sterilized by autoclaving at 121°C for 20 minutes¹⁵. Human osteosarcoma HOS cells (HOS, ATTC, and CRL-1543) were used to evaluate the toxicity and cell adhesion of the samples under study, these test were performed based on the procedures previously described in¹⁶. The HOS cells were cultured in Falcon culture plates and maintained in an incubator at a temperature of 37°C regulated with 5% CO₂ and saturated humidity. A RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% antibiotics was used as the cell cultured medium. For the cell toxicity test, the samples were placed in 1.5 ml of RPMI-1640 culture medium and incubated at 37°C. After 20 days, the culture medium was recollected and HOS cells were incubated with serial dilutions of each supernatant or medium alone for 72 hours at 37 °C. Cell toxicity was evaluated using a colorimetric MTT assay. For adhesion test, 1.5 x 10⁴ cells/ml in cultured medium were placed on each material and incubated at 37°C. After 120 hours, the cells were

detached using 0.5 ml Trypsin-EDTA for 5 minutes and counted microscopically in a Neubauer chamber. In addition, the cell morphology was reviewed using a fluorescence microscope (Nikon Eclipse E4000) equipped with a camera Nikon Coolpix 5000. Each test was performed three times and the results were calculated as number of cells per surface area.

2.6 Statistical Analysis

The distribution (normal/non-normal) of the numerical data and the statistical analysis to evaluate the influence of the voltage in the generation of the nanotubes was developed by Analysis of Variance (ANOVA), through the software Statgraphics. Values of $p \le 0.05$ are considered significant.

3. Results and Discussion

3.1 Morphological Features of the Anodic Layer

Figure 1 shows the formation of TiO₂ nanostructures at 14 V, 20 V and 25 V obtained by electrochemical anodizing.

Figures 1a, 1c and 1e show cavities of phase β (vanadium rich phase), where the nanotubes did not grow homogeneously due to the high solubility of the vanadium oxides formed in this phase¹⁷. On the other hand, in the Figure 1a, 1d and 1f it is possible to observe the morphology characteristics of TiO₂ nanotubes formed in electrolyte acid^{17,18}. In the SEM micrographs of the 14 V and 20 V experiments, an organized and homogeneous distribution of nanotubes was observed on the surface, with average diameter values of 54.35 nm and 90.84 nm respectively, shown in Table 1. These values are similar to those reported by^{19,20}, where TiO₂ nanotubes were developed and similar relationships were found between the voltage and the diameter of the nanotubes. In addition, in

Table 1. Average diameter of the nanotubes of TiO_2 obtained by electrochemical anodization in an electrolyte of 1M H₃PO₄ + 0.2% wt HF

Samples	Diameter (nm)	
14 V	54.35 ± 8.51	
20 V	90.84 ± 11.05	
25 V	85.02 ± 11.58	



Figure 1. SEM micrographs of TiO2 nanostructures obtained by anodizing at a) 14 V.50000X, b) 14 V. 300000X, c) 20 V. 50000X, d) 20 V.300000X, e) 25 V.50000X, f) 25 V. 300000X.

Source	Sum of Squares	Degreee of Freedom	Mean Squares	F	P
Between groups	84591.4	2	42295.7	385.90	0.0000
Within groups	35839.9	327	109.602		
Total	120431	329			

Table 2.	Analysis of Variance	(ANOVA) of the influence	of voltage on	the diameter	of the nan	otubes
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the SEM micrographs of the experiments at 25 V, a dual morphology is observed (formation of nanopores and nanotubes). The reason is the amount of voltage applied, causing a partial dissolution of the oxide layer, as reported by²¹.

A high voltage produces a fast dissolution of the electric fields, polarizing and weakening the Ti-O bond, not only in the lower part of the nanotubes but also through the whole length. The result is a random porous oxide structure as opposed to an organized one; this is recognized because there is no intertubular space between the pores as in the case of nanotubes. An organized and uniform structure was obtained for the anodized samples, with a nanotube density of $130/\mu m^2$, $60/\mu m^2$ and $6/\mu m^2$ for anodizing at 14 V, 20 V and 25 V respectively. In addition, for the conditions of 14 V, 20 V and 25 V, there were intertubular spaces of 10.98 nm, 14.20 nm and 14.27 nm respectively, increasing the surface area improving the transport of residues and nutrients and the cellular metabolism²². The voltage has a direct effect on the field-

assisted oxidation and dissolution reactions that occur during the formation of the nanotubular structures. The diameter of the nanotubes showed a significant difference for the different voltages applied in this study, which was determined by ANOVA analysis (Table 2), where the P-value of the F-test was less than 0.05, with a significant statistic difference among the means of the three variables with a 95% level of accuracy.

3.2 Electrochemical Measurements

Figure 2 shows the potentiodynamic polarization curves for the untreated sample (UT-Ti64) and the anodized samples at 14 V, 20 V and 25 V. The anodized samples showed a change of behavior on the cathodic zone, which can be attributed to the hydrogen production due to the reaction of the nanostructures with the compounds of the Ringer's solution²³. In addition, we noticed a change in the anodic zone of the anodized samples, which tends to passivate while the untreated sample (UT-Ti64) tends to the anodic dissolution. The anodized samples pres-



Figure 2. Potentiodynamic polarization curves for untreated Ti6Al4V alloy and UT-Ti64.

ent a lower corrosion potential than the UT-Ti64, this behavior is evidenced in a similar work by Hernandez-Lopez²⁴. Also, a lower corrosion rate in the UT-Ti64 is observed, generating a protective character as shown in Table 3. Figure 3 shows the Bode diagrams with impedance values (Figure 3a) and the phase angles (3b) of the different samples in function of frequency. The anodized sample at 14 V showed the highest impedance values, which is associated with a higher resistance to corrosion, while the



Figure 3. Bode diagrams for anodized samples and untreated titanium alloy. a) Impedance as a function of frequency in logarithmic scale, b) Phase angle as a function of frequency in logarithmic scale.



Figure 4. Equivalent circuits used to fit the impedance data for the (a) UT-TI64 alloy and (b) the anodic films grown.

Samples	E _{corr} (mV)	I _{corr} (μA/cm ²)	V _{corr} 10 ⁻³ (mpy)
UT-Ti64	-99	0.392	3.455
14V	-278	0.181	1.591
20V	-276	0.327	2.883
25V	-254	0.325	2.860

Table 3. Electrochemical parameters obtained for untreated Ti6Al4V alloy and anodized at 14 V, 20 V and 25 V

anodized samples at 20 V and 25 V, presented lower values of impedance than the untreated material (Figure 3a). This reduction is probably caused by the higher deposit of salts of the Ringer's solution on the surfaces of these samples due to a larger diameter of the nanostructure. In addition, it can be seen at high frequencies that there is a difference in the values of resistance to the solution, this can be due to the absorption of species by the nanostructures, which manages to generate an increase in the electrical resistance of the Ringer's solution. This species absorption indicates that the nanostructured TiO_2 surfaces are highly reactive compared to the untreated alloy. This could help the formation of calcium phosphates on this surfaces^{25,26}.

Figure 3b shows that at low frequencies (at the interface solution-nanostructured film) the anodized samples take values closer to 90° (more negative angles), compared to the untreated samples, being an indication of a capacitive behavior, originating the passivation of the film. In addition, the film shows a phase angle change from 7° to 20° in the high frequency range from 10 to 100 kHz. In the low frequency range from 0.01 to 100 Hz the phase angle increases from 35° to a maximum of 80°, showing a capacitive effect generated by the protective film; in the same frequency range, there is a linear relationship between the impedance and the frequency with a slope close to 1. For the case of samples anodized at 14 V the phase angle is in the range of 50-1000 Hz that is 65° and decreases to 45° in the low frequency range between 10 and 0.01 Hz due to the formation of double layer present in this type of coatings.

In the case of anodized samples at 20 and 25 V, there is a decrease of the phase angle to 55° in the frequency range of 1-100 Hz and reaches a value of 45° in the low frequency range of 0.1 to 0.01 Hz, we noticed less variation compared to the sample of 14 V, possibly due to the double layer formation. Figure 4 shows the equivalent circuits for the untreated samples and for the samples with nanostructured surfaces. The values obtained for the elements of the proposed circuit were calculated based on the experimental data obtained and simulated using the ZVIEW software. The equivalent circuit for UT-Ti64 is given by the Randles equivalent circuit, which consists of the resistance of the electrolyte (R1) in series with a constant phase element (CPE1) and a resistor (R2), characteristic of a resistive layer formed on the metal surface.

Table 4 shows the data for the untreated samples and for samples anodized at 14 V, 20 V and 25 V. It is observed that R1 (Solution Resistance) increases proportionally by increasing voltage, probably due to the absorption of salts on the surface of the anodized samples, which causes a decrease in the ion concentrations in the solution also causing an increase in the resistance. The resistance R2 for the anodized samples is the resistance of the interface solution-nanostructured film, R2 for the 14 V sample is much greater than the values obtained for samples at 20 V and 25 V, indicating a greater protective character, unlike the sample anodized at 25 V, which has a lower resistance. This is probably due to the presence of nanopores and nanotubes on its surface. The resistance R3 is

Element	UT-Ti64	14 V	20 V	25 V
R1	35.9	131.4	151.4	219.4
CPE1-T	1.31E-05	1.75E-06	9.80E-06	2.80E-06
CPE1-P	0.8702	0.8569	0.7546	0.8534
R2	1.98E+05	9052	3022	11.42
CPE2-T		1.36E-05	1.90E-05	2.13E-05
CPE2-P		0.5233	0.4148	0.4704
R3		5.35E+13	8.21E+06	1.60E+06
Chi Square	0.0087297	0.0001410	0.0002158	0.0006575

Table 4. Parameter values of the equivalent circuits for the untreated alloy and the anodic layers

usually attributed to the film formed on the metal substrate, also includes the charge transfer resistance due to the oxidation of the substrate, for this reason, R3 provides information on the corrosion protection that provides the film to the metal surface. The TiO_2 nanotube film formed at 14 V presented the highest value of R3, showing a better behavior than the nanostructured films formed at 20 V and 25 V.

The values obtained in the CPE1-P element indicates that the metal-solution interface and nanotubes-solution interface exhibits a highly capacitive behavior, which can be associated with the difficulty to transfer the charge due to the nanostructures of TiO₂ and the naturally formed titanium oxide on the surface of the alloy. On the other hand, the values obtained for the CPE2-P element, which is associated to the interface of the nanostructures with the substrate of Ti6Al4V, present a dual resistive-capacitive behavior, which is associated to the morphology of this interface. Finally, from the values of chi squared it can be inferred that are in the order of 1 x 10⁻⁴ indicating that the adjustments to the proposed equivalent circuit models are very good. Considering that the value of chi squared accounts for the difference between the experimental data and the data obtained from the circuit model.

3.3 Biological Test

None of the dilutions evaluated from supernatants samples incubated with RPMI medium achieved a percentage of toxicity equal to or greater than 10% in HOS cells. The results showed that all the samples allowed the adhesion of a quantifiable number of HOS cells by this method. It was found that the largest number of adhered cells was obtained in those of the 25 V samples. Most of the replicates of the 14 V and 20 V samples showed higher values than those obtained for UT-Ti64 control samples. On the other hand, the percentage of cellular viability varies indistinctly among all the samples; however, the samples with the highest number of cell adhered to its surface showed higher percentages (80% for 20 V and 25 V samples). Figure 5 shows a summary of the results for cell adhesion to samples.

Figure 6 shows the cells adhered to the surface of the samples using fluorescent compounds like phthalocyanine aluminum and hoesch 33342. Evaluation of cellular adhesion using a fluorescence microscopy showed the HOS cells on the surface of the different samples. The dyes used allowed to observe the cytoplasm (Red) and the core (Blue) of the cell. These attached cells, were of elongated and uniform appearance, did not show nuclear alterations like condensation or fragmentation of the DNA that can indicate death or cellular damage. Besides, a smaller amount of cytoplasm was found in the MB samples compared to the 14 V, 20 V and 25 V specimens. Which may indicate a better adhesion to these surfaces? The adhesion/ propagation of the osteoblasts is significantly improved by the presence of TiO₂ nanotubes with diameters of 20 to 120 nm with the filopodia of the osteoblasts growing in the pores of the nanotubes and forming an interlaced cell structure. Also the chondrocyte adhesion increases by 40% on the surfaces with TiO, nanotubes with internal diameter of 70-80 nm compared to surfaces without nanotubes as evidenced by²⁷. This may indicate that pore sizes of



Figure 5. Biological tests. a) Cell adhesion, b) Cell viability.



Figure 6. Adhesion by fluorescence microscopy. a) UT Ti64, b) 14 V, c) 20 V and d) 25 V.

60-80 nm obtained in this research could favor the growth and storage of the nanotubes and improved cell adhesion.

4. Conclusions

- TiO₂ nanotubes with diameters of 54.35 nm, 90.84 nm and 85.02 nm were obtained by anodizing at 14 V, 20 V and 25 V respectively. However, for the 25 V condition, a dual morphology of nanotubes and nanopores was obtained.
- Using an anodizing process, an organized and uniform structure was obtained with a density of 130/µm², 60/µm² and 6/µm², for the samples anodized at 14 V, 20 V and 25 V respectively, evidencing an increase of the surface area for the samples of 14 V.
- The anodized samples presented nanotubes with intertubular spaces between 10 and 14 nm, favoring the transport of residues and nutrients and, therefore, the cellular metabolism.
- The nanostructures obtained by the anodizing process presented a lower corrosion rate compared to the unmodified samples. In this sense, the samples anodized at 14 V were those who presented the angle closest to 90° favoring a

capacitive behavior, which causes the passivation of the film.

• The anodized samples presented higher cell adhesion compared to the base material. On the other hand, anodized samples at 20 V and 25 V presented 80% cell viability compared to 20% of samples anodized at 14 V.

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