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Titanium-Tantalum Alloy Surface Modification by Hydroxyapatite Layer on TiO_2 Nanotubes: Effect on Microbial Activity

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One of the major health security challenges of the 21st century is the occurrence of microbial infections and bacterial complications that could affect 10 million people by 2050. On the biomaterial field, implant metallic currently replaces partial or total body parts and can fail to be integrated into the body due to infections. This study performs two combined surface modifications on Ti-30Ta alloy, in order to obtain an infection-resistance and osseointegration surface on metallic implants to be tested within bacterial biofilm. The Group 1 investigated surface modifications by the anodization process in the electrolyte glycerol + NH_4F 0.25% at 30V- 9 hours and annealed in 530°C (5°C/min). The Group 2 underwent the same process as Group 1 and, additionally, the samples were immersed in 0.3 M $CaCl_2$ and 0.5 M Na_2HPO_4 solutions for hydroxyapatite growth. The substrate was characterized using scanning electron microscopy (SEM), X-ray diffractometer (XRD) and dynamic contact angle. S. epidermidis bacterial adhesion and biofilm formation. The results indicated that the Group 1 shows a higher antimicrobial activity, hydrophilic behavior and potential to be used for metallic implant applications. The Group 2 with the hydroxyapatite film coating did not have an improvement in the antimicrobial response.

Keywords: *Titanium alloy, surface modification, antimicrobial activity, nanotubes coating, Hydroxyapatite.*

1. Introduction

One of the major health security challenges of the 21st century is the occurrence of microbial infections. This challenging scenario is difficult to overcome due to the antimicrobial resistance (AMR) identified in some microorganisms. Therefore, it is expected that 10 million people will have infections-related complications by 2050 and this imposes a huge social and economic burden worldwide[1,2].

Staphylococcus epidermidis is a member of the normal human microbiota, usually found on the skin and mucous membranes. This microorganism adheres to the surface of the host tissue by specific adhesion mechanism, establishing a continuous commensal relationship with humans. It is noticed that *S. epidermidis* provides some benefits to the human host, by the competition with other virulent pathogens[3]. However, mainly due to its capability to form biofilm in implanted foreign bodies, *S. epidermidis* has arisen as an important opportunistic pathogen in patients that receive medical devices[4]. Indeed, *S. epidermidis* is one of the most commons bacteria that cause infections related to the orthopedic devices[5].

Multicellular aggregates, known as biofilms, are formed due to several intrinsic and extrinsic bacteria factors. For the formation of biofilm, bacteria produce an extracellular matrix composed of carbohydrates, proteins and/or extracellular DNA. This viscous structure facilitates survival of microorganisms in hostile environments. In addition to the better adhesion provided by an extracellular matrix, biofilm gives bacteria greater resistance to the immune system response and to antimicrobials[6,7].

Extracellular matrix and the infections associated with biofilm are difficult to treat, mainly because the biofilm blocks the entrance of antimicrobial drugs into the bacterial site[8,9]. The biofilm of bacteria comprises microorganisms in which cells stick to each other and also to a surface[10]. In this context, non-traditional antimicrobial agents have been identified as hopeful tools against resistant bacteria to traditional antibiotic

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drugs[11]. One kind of non-traditional antimicrobials newly introduced is nanomaterials. Nanomaterials demonstrate toxic effects against several bacterial strains during *in vitro* studies and appropriate biocompatibility properties to the human body. Because of these results, nanomaterials could be promising in several biomedical applications[12].

Implantable medical devices provide a solution to partial or total replacement of body parts. Also, many implants are made with metals that are susceptible to bacteria contamination and provide ideal surfaces for biofilm formation that results in severe infections leading to implant failures[13-15]. Recent projects have been evaluating titanium-alloy implants that enhance surface-mediated bone formation and the capability to control infection simultaneously without medicines. Further increasing bone integration, a number of surface modification strategies have been investigated. Techniques of surface modification on nanoscale show osseointegration improvement and antimicrobial activity[16].

Titanium and its alloys do not induce any biological response themselves, because they are biologically inert or bioinert. They might exhibit ion release and rather leading to fibrous encapsulation. Poor tissue-material integration is the major cause for long-term implant failure. In order to make them biologically active and mitigate the described issues, the material surface undergoes through treatments to induce response onto implant surroundings. There are many different surface treatments, as sorted in mechanical and chemical surface modification. Mechanical modifications aim to change the material topography, in other words, they either remove or shape the surface. Modifications such sanding, polishing and particle blasting[17] are basic-method examples. Chemical modifications are the mostly applied in surface treatments. Many different methods feature chemical modifications, such as acid corrosion[18], sputtering by ion-beam-assisted deposition[19,20], sol-gel incorporation[21], electrolysis[22], electrochemical anodization[23,24], ion implantation[25] and biomimetic[26].

A common surface treatment towards titanium and its alloys is anodization. This process aims to texturize the surface with patterned titanium oxide layer by passing current through an electrolytic cell, composed by a counter-electrode, which may be made of platinum, gold, carbon and among other noble metals; a working electrode, which is made of the titanium that interacts within the media; and an electrolyte, which is the media itself. Different results, such as nanotubes, can be achieved by anodization. Their structures depend on the media composition and the electrical current and tension parameters to correctly texturize these alloys[27,28]. These nano-texturing has shown an increase of cells growth and healing process, as well as a decrease of microbiological pathogens proliferation that might infect the implant area[29].

Another important surface treatment is the immobilization of hydroxyapatite. It has titanium-surface bioactive properties that mimetic the organism. Over the last three decades, scientific experiments has shown the effects of this surface modification. The functionalization process starts by soaking the samples in NaOH alkaline solution and then evaluating them in simulated body fluid (SBF)[30]. The hydroxyapatite components are phosphate and calcium, present in bones. Therefore, the similar composition of the implant surface with bones stimulate the adhesion and growth of cells over the implant[31]. However, hydroxyapatite does not show an antimicrobial response by itself. Thus, it requires to be doped or synthesized with other antimicrobial components[32].

It is important to highlight that the treatment of the implant surface has an intrinsic relation to its contact angle. The contact angle can distinguish the characteristic of the surface as hydrophilic (angle < 90º) or hydrophobic (angle > 90º). Hydrophilic surfaces present higher cell adhesion. Additionally, the dynamic contact angle analysis gives a better assessment than the static one, because it provides more information of the surface under a flow rather than under a static droplet. In other words, it represents more realistically the surface behavior in a dynamic scenario like for implants in human bodies[33,34].

The surface modification treatment can improve the characteristics of implantable devices. It may prevent the occurrence of microbial growth, increase protection against ions liberation, stimulate cellular growth, and increase mechanical features, among many other improvements. Research projects assess how the different combined modifications can foment better healing mechanisms to patients[35-37].

Likewise, this work aims to analyze two surface treatments that consist in use anodization processes to obtain nanotubes and hydroxyapatite coatings over Ti-30Ta samples to evaluate their structural features and antimicrobial activity against *S. epidermidis* biofilm formation.

2. Materials and Methods

2.1. Ti-30Ta Alloy processing

To obtain the Ti-30Ta alloy, commercially pure metals were used in sheets: titanium (Sigma-Aldrich) and tantalum (Sigma-Aldrich). The Ti-30Ta alloy was melted in an arc furnace with an inert atmosphere.The ingots were homogenized under vacuum at 1000ºC for 24 h to eliminate chemical segregation. Then the samples were cold-worked formed in such a way that there was a decrease in the diameter of the sample in the order of 20%. The rotary swaging was performed on FENN equipment models 6F (2" to 3/8") and 3F (1/2" to 1/8"), with a speed of 1700 revolutions per minute (rpm), 30 CV (HP). The bars were subjected to the solubilization heat treatment at 950° C for 2 hours, followed by rapid cooling in water. The bars were cut into discs of 10 mm of diameter and 3 mm of thickness[38].

2.2. Surface treatment

All substrates were ultrasonically cleaned with distilled water and acetone. The Ti-30Ta alloy substrates were sorted into two groups for this study: Group 1, which consists in substrates anodized in order to obtain TiO_2 nanotubes; and Group 2, which consists in hydroxyapatite immobilization, after the anodization process, following the methodology described by Lett et al.[39].

2.2.1. Group 1 - TiO_2 nanotubes

The anodization process was performed on the substrates for Group 1 using a dual electrode system with platinum (counter

electrode) (Sigma) and the Ti-30Ta alloy (working electrode), attached at 15 mm apart. The electrodes were connected to a power supply (Fisher Scientific FB300 Electrophoresis) in an electrolyte made of 0.2 M NH_4F and glycerol at 30 V for 9 hours. Following the anodization process, the Ti-30Ta alloy substrate was rinsed in isopropyl alcohol and dried by compressed air. All anodized substrates were further annealed in an oxygen ambient furnace at 530 ºC, with a ramping rate of 5 ºC/min for 1 hour[24].

2.2.2. Group 2- TiO_2 nanotubes with hydroxyapatite

For Group 2, all samples passed previously by the anodization process just like in Group 1. In sequence, the immobilization of hydroxyapatite was carried out by using two solutions: 0.5 M calcium chloride and 0.3 M disodium phosphate. Following the protocol indicated by Lett et al.[39], the samples were immersed in 0.5 M calcium chloride (Merck) solution for 10 minutes, rinsed in deionized water for 1 minute, immersed in 0.3 M disodium phosphate (Merck) during 10 minutes and finally rinsed for another 1 minute in deionized water. This step consists in one cycle out of three-cycle routine. However, during the second and third cycles, the samples were immersed during 5 minutes instead of 10 minutes in each solution. After mineralization, the samples were kept in a desiccator for future analysis.

2.3. Characterization

The samples were characterized by SEM imaging (JEOL JSM 6100) and the substrates were coated with 10 nm of gold and imaged at 15 kV. The elemental surface composition was characterized by energy-dispersive spectroscopy (EDS, JSM 6100 SEM). The material surface analysis provided a complete profile of the different present elements. Spatial element mapping was performed by grouping pixels with similar atomic spectra. The crystallinity of the samples was investigated by X-ray diffraction analysis (XRD), using X' Pert Philips PMD with a Panalytical X'celerator Detector (Malvern), using a CuKα radiation and λ = 1.5418 A. The wettability of the substrate surfaces was investigated by using a sessile drop method (2 ml) with a contact angle goniometer (Kruss DSA 10) equipped with video capture. The resulting images at the water-substrate interface were fit using the circle fitting profile.

The wettability of the substrate surface was investigated by Dynamic Contact Angle (DCA) analysis. The equipment CA-2500 XE™ (AST products Inc.) performed immersion and emersion movement in force loop following the Wilhelmy method, following the Equation 1 that is an alternative form of the fundamental Young equation, in which $\gamma_{lv}cos\theta$ represents the equilibrium wetting energy, being F the force, l the length variation, γ_{lv} the surface tension of the liquid and θ the contact angle.

$$\frac{F}{l} = \gamma_{lv} l cos\theta \quad (1)$$

The advance and reactance data from DCA experiment provided information to calculate the hysteresis of wetting tension. In other words, hysteresis ($\Delta\theta$) is the difference between the advancing and receding angles[40,41]. Three loop assays evaluated the wettability of Ti-30Ta surface covered by nanotubes in order to compare them with the sessile drop method.

The bacterial proliferation analysis was performed according to the methodology proposed by Pereira (2011)[42] using the reference strains [American Type Culture Collection (ATCC)], *S. epidermidis* (ATCC 6538). The strains were seeded in agar brain heart infusion (BHI) and incubated at 37 ºC for 24 hours. Then colonies of microorganisms were suspended in sterile physiological solution [0.9% sodium chloride (NaCl)] and adjusted to 0.5-turbidity on the MacFarland scale, 1.5 × 108 of colony forming units per milliliter (CFU/ml).

The samples were organized in three groups: Ti-30Ta (control), Ti-30Ta coated with nanotubes and Ti-30Ta coated with nanotubes and hydroxyapatite. All samples were placed in 24-well plates with 2 ml of BHI broth supplemented with 5% sucrose and inoculated with 0.1 ml of the bacterial suspension. The samples were incubated at 37 ºC for 48 hours and the media was replaced after 24 hours. After that period, the samples were washed aseptically with 2 ml of sterile physiological solution, placed in tubes with 10 ml of sterile physiological solution and sonicated for 30 seconds to disperse the biofilms. The suspension was considered, as dilution factor, 10^{-1} and diluted with the addition of sterile physiological solution to 10^{-8}. Aliquots of 0.1 ml were seeded on BHI agar plates and incubated for 24 hours at 37 ºC. The number of colonies was counted, calculated the CFU/ml and transformed in $\log_{10}$[42].

Each experiment was reconfirmed on at least three substrates (n = 3). All the quantitative results were analyzed using an Analysis of Variance (ANOVA). Statistical significance was considered at $p < 0.05$. During the analysis, variances among each group were not assumed as equal and a two-sample t-test approach was used to test the significance between the Group 1 (TiO_2 nanotube) and Group 2 (TiO_2 nanotube + hydroxyapatite). This analysis was done using the Microsoft Office Excel data analysis software.

3. Results and Discussion

The results obtained for both groups followed the methodology described above. Initially, the nanostructures of the coatings had to be evaluated before the characterization of their properties. Thus, the Group 1 SEM micrographs, on Figure 1, confirmed the growth of nanotubes on 0.2M NH_4F and glycerol electrolyte at 30V for 9 hours with 80-100 nm of diameter covering all the surface. Furthermore, in the Group 2, hydroxyapatite film was successfully coated on the TiO_2 nanotubes. The Figure 1, Group 2 shows the effectiveness of hydroxyapatites formations by using the described methodology. Biomimetic coating technique can be employed to deposit calcium phosphate on substrate. Further analysis of both coatings (Figure 2) with EDS confirmed the elemental composition of calcium and phosphorus, indicating that the coating was composed by Ca–P phase. Mesenchymal stem cell investigation on hydroxyapatite surface presents better cell spreading than in a regular alloy surface[43,44].

Figure 3 shows the XRD spectra of Groups 1 and 2. The results indicate that on the Group 1, after being applied 30 V for 9 hours, the anodic film process produces TiO_2 anatase phase in this experiment and the Group 2 shows the same

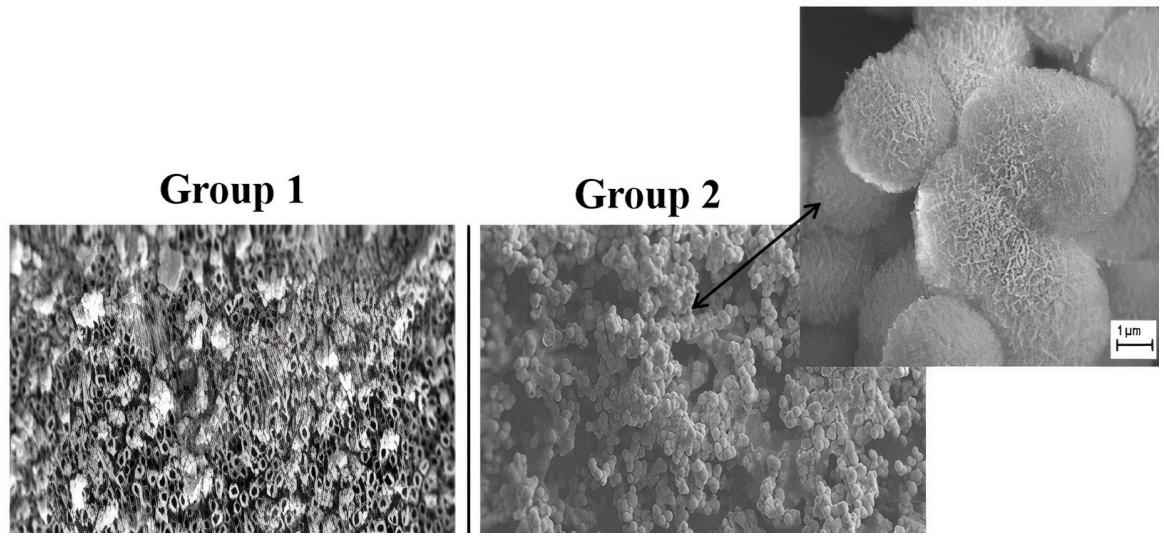

Figure 1. SEM images of Gorup 1 (TiO_2 nanotube) and Group 2 (TiO_2 nanotube + hydroxyapatite). The projected detail shows hydroxyapatite at higher magnification.

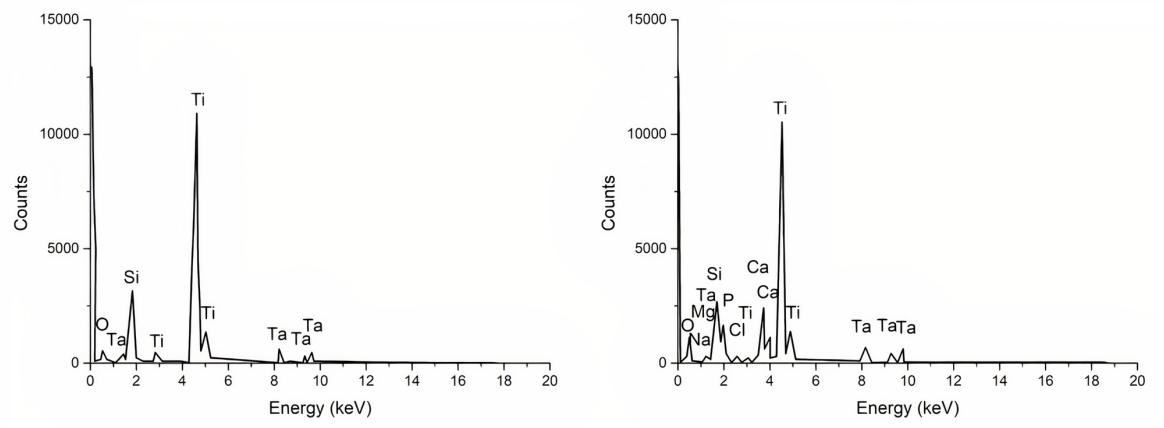

Figure 2. EDX spectra of Group 1 (TiO_2 nanotube) leftwards and Group 2 (TiO_2 nanotube + hydroxyapatite) rightwards. Peaks of calcium (Ca) and phosphorus (P) confirms hydroxyapatite covering.

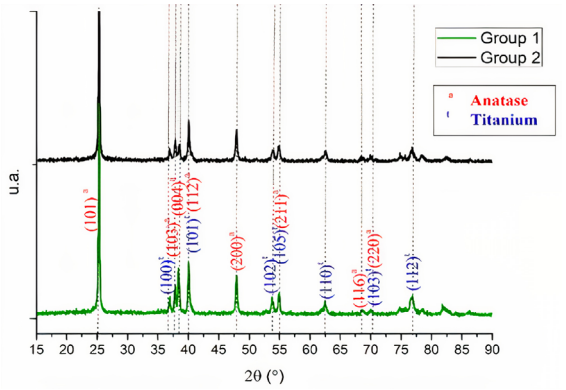

Figure 3. XRD pattern of Group 1 (TiO_2 nanotube) and Group 2 (TiO_2 nanotube + hydroxyapatite). Both groups present anatase phase with peaks at 25°.

results. Thus, it is possible to state this by comparing the anatase XRD patterns of both groups. The anatase is a desirable TiO_2 phase to metallic implants. Several studies have demonstrated the excellent biocompatibility properties due to the increase of cellular response and consequently the osseointegration[16,45-47].

In this study, nanotubes of TiO_2 and nanotubes of TiO_2 + hydroxyapatite coating grown on the Ti-30Ta alloys surface were successfully obtained. Bacterial proliferation was investigated by SEM images of Group 1 and Group 2. Furthermore, the number of CFU/ml of *S. epidermidis* was identified after the biofilms have grown incubated at 37 °C for 48 h (Figure 4). Figure 4a shows the resultant bar-graph value of CFU/ml for *S. epidermidis* in Group 1 and Group 2. The results are presented as the mean ± standard error mean from 10 samples per group (* $p < 0.05$). The CFU/ml in the Group 2 is similar

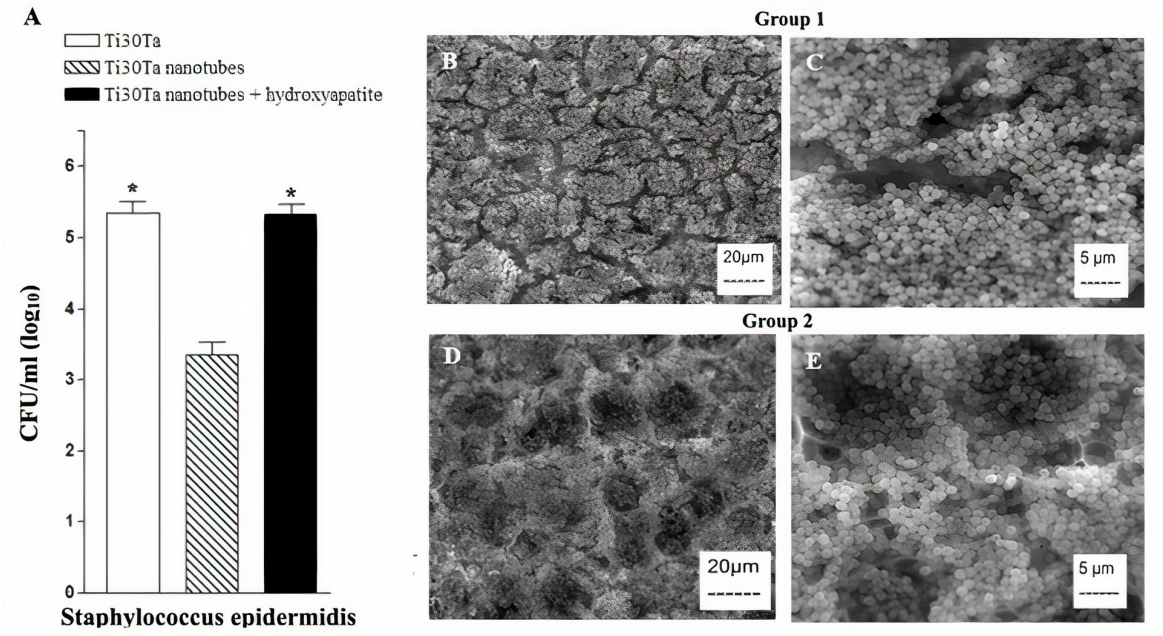

Figure 4. SEM images of Group 1 (TiO_2 nanotube) and Group 2 (TiO_2 nanotube + hydroxyapatite); a) graphic relationship of CFU/ml for each group, b) image with lower magnification for Group 1, c) image with higher magnification for Group 1, d) image with lower magnification for Group 2, e) image with higher magnification for Group 2.

to the value registered for the control Group (samples of Ti-30Ta) and is statically higher than in the Group 1. This demonstrated the lower antimicrobial response of the Group 2 when compared to the Group 1. Group 1 coating presents nanoscale level because of TiO_2 nanotube diameter range between 80-100 nm. The Group 2 coating shown a circular hydroxyapatite shape with 4-6 µm diameter. Clearly, the nanoscale surface has strong antimicrobial efficacy by inhibiting the *S epidermidis* growth[46,48-53]. In previous studies, it was investigated the behavior of gram positive and negative bacteria on TiO_2 nanotubes. The nanometric surface also showed antibacterial activity compared to flat surfaces[54,55]. In opposition, another study applied efforts to dope hydroxyapatite with antimicrobial agents because it does not have an innate response for microbial formation, despite its abroad use for bone regeneration[32].

Examination of the samples by SEM showed biofilm formation on the surface of the coating Group 1 (TiO_2 nanotube) and Group 2 (TiO_2 nanotube + hydroxyapatite) in lower magnification in Figure 4b and 4d and higher magnification in Figure 4c and 4e. Groups 1 and 2 images are morphologically similar, despite the number of CFU/ml that is statistically significant. On both groups, the surface was covered with *S. epidermidis* microorganism consisting of nonmotile gram-itive *cocci* and arranged in grape-like clusters. Also, the colonies raised and cohesive about 1–2 mm in diameter.

The wettability definition is the ability of liquids to keep in contact with solid surfaces due to a direct result of intermolecular interactions between liquid and solid. The degree of wettability is determined by force balance between adhesive and cohesive forces and it is measured by a contact angle (CA). CA < 90° is denominated hydrophilic and the fluid spread over a large area of the surface. In addition, for CA > 90°, the fluid minimizes the contact with the surface and form a compact liquid droplet. It is addressed to hydrophobic surfaces[56].

The antimicrobial activity of the surface presents intimate relation with the wettability. Therefore, the contact angle was analyzed for Group 1 and it presented a hydrophilic surface, as well as the Group 2. The Group 1 presented CA of 32.52° ± 1.86º, being higher than the Group 2 (15.62° ± 2.47º). Without any coating, the contact angle registered was 65,89º ± 1,81º. Thus it is possible to assume that the surface treatment impacted on the wettability of the material decreasing the contact angle, even though it has kept it as hydrophilic.

The dynamic contact angle was used to evaluate and confirm the wettability of nanotube surface modification. This analysis focused on the Group I because it had a better response as an antimicrobial surface than the Group II. The technique brings in information of the surface in movement. It analyzes the advance and recede of surface in a flow. Two different angular registers of the wettability are made during the controlled movement stress. This movement provides wider information than the static technique. The Figure 5 presents a graph that follows the Wilhelmy method[57], taking γ_{lv} for pure water, 72.5 mN.m^{-1}. The graph shows some hydrophobic conditions during the evaluation (values < 0). However, the receding movement and some of advancing movement present values higher than zero, meaning hydrophilic behaviors predominantly[58-60]. By calculating the $\Delta\theta$, it was found an angle of 40.53°. This value approaches to the sessile drop method, when both showed hydrophilic angles. The difference can be justified by the water purity.

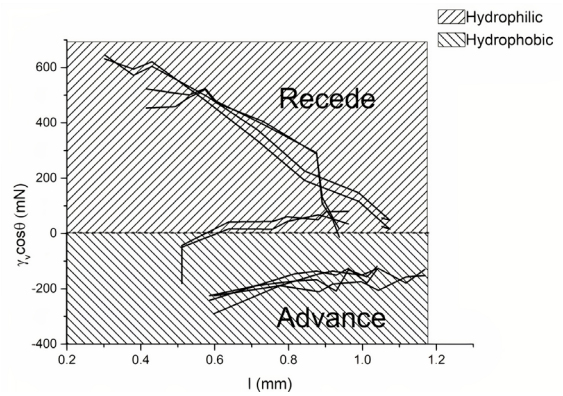

Figure 5. Graph following Wilhelmy equation that describes the loop movement of advancing and receding dynamic contact angle in hydrophilic and hydrophobic regions.

Therefore, the results indicated that the Ti-30Ta surface coated with nanotubes is more suitable for practical applications. The presented hydrophobicity presents high influence on cell adhesion behavior. The hydrophilic surface enhances the cell anchorage, being more effective to stabilize the modified material[61]. The This point is argued due to the better antimicrobial response achieved by the Group 1 when compared to the Groups 2 and control, as well as its hydrophilicity, identified in the dynamic contact angle analysis, that can enhance the cell adhesion over the implant.

4. Conclusion

Through Ti-30Ta alloy surface microscopy techniques, it is possible to see that the surface modification by growing *in situ* TiO_2 nanotube by anodization process, and then, the hydroxyapatite film covering the nanostructured surface. The diameter of nanotube range 80-100 nm and the hydroxyapatite, 4-6 µm.

The TiO_2 nanotube and TiO_2 nanotube + hydroxyapatite coating present XRD spectra similar to the TiO_2 anatase phase, being important to cell adhesion. The wettability results presented hydrophilic surfaces for both groups. Yet, the dynamic contact angle was confirmed by a different analytical method the hydrophilic surface feature. This is also relevant for cell adhesion in implanted devices.

The antimicrobial activity on the surface of anodized materials covered with TiO_2 nanotube is higher than in hydroxyapatite film. In other words, the Ti-30Ta alloy surface modified by anodization process exclusively had a higher antimicrobial activity. Thus, this material and its nanostructured coating present a potential method to enhance the surface of metallic implants for practical applications.

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