Materials Research. 2023; 26: e20230113
DOI: https://doi.org/10.1590/1980-5373-MR-2023-0113

Surface Modification of the Ti25Ta25Nb3Sn Alloy and its Influence on the Cell and Bacteria Adhesion – In Vitro Studies

Marisa A. Souza[a], Marcia C. M. Lima[b], Ana L. A. Escada[a], Bruna N. A. S. Pimentel[c], Carlos E. Vergani[c], Roberta M. Sabino[d], Ketul C. Popat[d], Ana P. Rosifini Alves[a]*

[a]Universidade Estadual de São Paulo (Unesp), Faculdade de Engenharia e Ciências, Departamento de Materiais e Tecnologia, Guaratinguetá, SP, Brasil.
[b]Universidade Estadual de São Paulo (Unesp), Instituto de Ciência e Tecnologia, São José dos Campos, SP, Brasil.
[c]Universidade Estadual de São Paulo (Unesp), Faculdade de Odontologia, Departameto de Odontologia e Prótese, Araraquara, SP, Brasil.
[d]Colorado State University (CSU), Department of Mechanical Engineering, 80523, Fort Collins, CO, USA.

Received: January 27, 2023; Revised: April 28, 2023; Accepted: June 19, 2023

In this study, alkaline treatment was used to change the surface of the new beta titanium alloy Ti25Ta25Nb3Sn and enhance its bioactivity. Ti25Ta25Nb3Sn, a new experimental alloy was processed in the laboratory and the surfaces were modified using alkaline treatment. Samples were soaked at 60 ^{0}C for 24 hours in different NaOH solutions to obtain surfaces with different morphologies. The surfaces were characterized by different techniques such as X-ray diffraction (XRD), scanning electron microscopy (SEM), and Atomic Force Microscopy (AFM). The wettability of the surfaces was measured using the sessile drop method. In vitro studies were carried out to evaluate cell adhesion, proliferation, and bacteria adhesion. As a result of the alkaline treatment, a nanoporous and amorphous layer was formed on the Ti25Ta25Nb3Sn alloy surface with super hydrophilic behavior for all conditions evaluated. However, different nano topography was observed which affected biological response. The morphology obtained after immersion in 1.5 NaOH solution led to bacteria adhesion decreased and osteoblasts cells differentiation. Results of this study show that is possible to obtain a new titanium experimental alloy with excellent bulk and surface properties that may decrease the risk of infections and increase osseointegration.

Keywords*: titanium alloys, surface treatment, alkaline treatment, cell adhesion, nanoporous layer.*

1. Introduction

Metallic materials, such as titanium and its alloys, are used as bone substitutes due to their mechanical properties, excellent corrosion resistance, high yield, good ductility, low density, high wettability, and biocompatibility in the manufacture of dental implants. However, most titanium-based materials have an elastic modulus higher than that of human bone[1]. Due to this limitation, in recent years, titanium-based alloys with different compositions have been studied for biomedical applications[2-7].

Recently, Ti-25Ta-25Nb-3Sn[8] was studied by our group and showed excellent mechanical properties, highlighting its low elastic modulus. However, in addition to the low elastic modulus, another important factor is the surface of the material. It should be directly linked to the response in the bone/implant interface and osseointegration. Despite their biocompatibility, titanium, and titanium alloys are classified as bioinert materials. This means that when they are inserted into the human body, they do not induce a response from the surrounding tissue, as occurs with bioactive biomaterials. Bioinert materials may exhibit the formation of a non-adherent fibrous layer around the implanted component, preventing any direct interaction with the recipient tissue. In this case, the bond between these materials and the bone tissue is purely mechanical, which requires a long period of osseointegration and can often lead to movement at the bone-implant interface[9,10].

In recent years, several studies have been done with the goal of accelerating osseointegration through surface modification. The titanium surface's mechanical, chemical, and physical modification techniques can be used to increase bioactivity, biocompatibility, blood compatibility, wear, and corrosion resistance. There is also a tendency to replace mechanically treated surfaces with chemically modified surfaces for better surface response[11].

Chemical methods include several techniques, such as sol-gel coatings, chemical vapor deposition, biochemical modifications, electrochemical anodization, and alkaline treatment[12-16]. Alkaline treatment is a widely used chemical method to improve the bioactivity of titanium and its alloys, and it was introduced in the 1990s by Kim et al.[16]. This

*e-mail: paula.rosifini@unesp.br

method is based on the immersion of the material in NaOH with variations in molarity, temperature, and time. It induces surface modification by allowing the growth of a bioactive nanostructured sodium titanate layer on the titanium surface. The sodium titanate formation appears due to the presence of the hydroxyl groups (OH), which dissolve the TiO2 passive layer and produce negatively charged hydrates (HTiO3-). This negatively charged surface incorporates Na+ ions and produces a hydrogel layer of amorphous sodium titanate (Na2Ti5O11 or Na2Ti6O13), which stabilizes into crystalline sodium titanate after heat treatment[17]. Since then, some researchers have found that a stable layer of sodium titanate (amorphous) forms on the surface of titanium after alkaline treatment[18].

In addition to creating a bioactive surface, alkaline treatment is considered a preliminary step for biomimetic treatment. It acts as a catalyst for subsequent calcium phosphate nucleation, mimicking the initial surface reaction after implantation[19].

In a simulated body fluid (SBF) solution, this bioactive sodium titanate layer releases its sodium ions (Na+) and forms Ti-OH groups through ion exchange with the H3O+ ions present in the fluid. The TiOH groups interact immediately with calcium ions (Ca2+) to form calcium titanate, which incorporates the phosphate ions (PO42-), and various types of calcium phosphates are formed[20].

Also, the alkaline surface treatment directly affects the surface wettability, leaving it hydrophilic. Surface wettability can affect four important aspects of the biological system: 1) protein adhesion; 2) interactions of soft and hard tissues with surfaces; 3) bacterial adhesion and biofilm formation; and 4) osseointegration rate[21]. A study comparing Ti implants with alkaline treatment and acid treatment showed that the alkaline treatment made Ti implants superhydrophilic (contact angle of almost 0 degrees), resulting in much faster and more extensive surface mineralization when compared to Ti implants with acid treatment[22].

The sodium hydroxide treatment also increased the hydrophilicity on the surface of Ti plates. In addition, it increased the adhesion and proliferation of human periodontal ligament fibroblasts[14,23].

Hence, the objective of this study was to modify the surface of the Ti25Ta25Nb3Sn, a new beta titanium alloy, using alkaline treatment with varying NaOH concentrations to achieve a bioactive surface. Subsequently, these samples were characterized by SEM, X-ray diffraction, and contact angle. They were evaluated by in vitro studies for cell adhesion and proliferation, as well as bacterial assays.

Thus, the development of new antibacterial agents, towards which bacteria cannot develop resistance, is a pressing issue. For the case of orthopedic implants, the development of new implant materials that prevent attachment and growth of bacteria would certainly decrease the number of failed orthopedic implants and improve the quality of life for patients.

2. Materials and Methods

2.1. Sample preparation

The Ti25Ta25Nb3Sn alloy was produced from sheets of commercially pure titanium (99.5%), tantalum (99.5%), niobium (99.9%), and tin (99.9%). Sheets were melted in an arc melting furnace under an argon atmosphere and re-melted at least ten times to ensure homogeneity. The ingots were homogenized under a vacuum at 1100°C for 24 hours to eliminate chemical segregation. They were then cold rolled using rotary swaging at room temperature, resulting in a final diameter of about 10 mm, before being heat treated. Samples for this research, with a thickness of 6 mm, were cut from the ingots, sanded (using sandpaper #100), and cleaned with an ultrasonic bath containing acetone for 30 minutes.

2.2. Alkaline surface treatment

Alkaline treatment methodology was based on our previous study with Ti30Ta[12]. Samples were immersed in a 0.5 M, 1.0 M, and 1.5 M NaOH aqueous solution at 60°C for 24 hours, washed with deionized water, and dried at 40°C for 24 hours. After alkaline treatment, they were heat-treated at 300°C for 1 hour in the air in an electric furnace.

The surface morphology of the samples before and after alkaline treatment was evaluated using scanning electron microscopy (SEM-FEI Magellan 400L, Hillsboro, OR). The surface topographic images were recorded using an atomic force microscope (AFM) and Nanoscope III, using contact mode. All the images were recorded under an air atmosphere at room temperature.

Surface wettability measurements were also conducted. Contact angle measurements were obtained using an automated goniometer (Krüss DSA 100S, Krüss Company Ltd., Hamburg, Germany). A water drop of 10 µL was added, and the average of three readings was taken for each group's three samples.

2.3. In vitro studies

2.3.1. Cell culture

Cells were removed from the rat bone marrow and cultured at 37°C and 5% CO2 in growth media consisting of MEM-Alpha Modification Media (Invitrogen, 12571-063) with 10% Fetal Bovine Serum (F2442) and 1% penicillin/streptomycin (Invitrogen, 15140-148) (5 mL of 100X for 500 mL media). For cell viability, the reading was taken using a spectrophotometer with Omega software.

Adhesion and proliferation were evaluated by fluorescence microscopy after 4 and 7 days using Rhodamine Phalloidin (F-actin stain-cytoskeleton). The cells were fixed with 3.7% formaldehyde (5 mL of 37% formaldehyde in 45 mL of PBS) for 15 minutes at room temperature. They were then incubated with 300 µl of 1% Triton and 300 µl of Rhodamine actin for 25 minutes. Finally, DAPI was added for 3 minutes.

The morphology of cells adhered to the surfaces was evaluated using scanning electron microscopy (SEM) after 4 and 7 days of culture (Philips XL-30 FEG) in 30% glutaraldehyde, 1.68 g cacodylate, 2.72 g sucrose, and 77.6 mL of distilled water. After fixation, the cells were dehydrated in ethanol (35%, 50%, 70%, and 100%). The samples were placed on a glass plate and treated with 100% hexamethyldisilazane (HDMS - Sigma) for 10 minutes. The surfaces were coated with 10 nm of gold using the Balzers Sputter Coater SCD 004.

2.3.2. Bacterial analysis

A reference strain of Staphylococcus aureus (ATCC 25923) was used in this study. The strain was seeded on Miller

Hinton agar (Kasvi, Brazil) and incubated at 37°C for 48 hours (MA 0324, Marconi Laboratory Equipment Ltda; Brazil). For this purpose, eight colonies were transferred to 10 mL of Tryptic Soy Broth (TSB; Accumedia Manufacturers, USA) culture medium, forming the pre-inoculum, and incubated overnight (18 hours) at 37°C. Then, 1.5 mL of the pre-inoculum was transferred to a new Falcon tube containing 28.5 mL of fresh TSB medium, forming the inoculum, and incubated for 4 hours at 37°C. The microorganism suspension was centrifuged (4000 xg for 5 minutes at 4°C; 5810R, Eppendorf AG, Germany), and the pellet was washed with PBS and resuspended in TSB culture medium. Sterile specimens were packed into a 24-well plate (TPP Techno Plastic Products, Switzerland), and 2 mL of inoculum was added to each well. For microorganism adhesion, the plate was incubated with an orbital shaker (37°C, 75 rpm; Q816M20, Quimis, Brazil) for 90 minutes. After this period, two washes were performed with 1 mL of PBS to remove the non-adhered microorganisms. Then, 2 mL of TSB medium was added to each well, and the plate was incubated with an orbital shaker for 48 hours with medium exchange after 24 hours.

For quantitative analysis, after the period determined to obtain the biofilm, the specimens were carefully washed three times with PBS (for removal of unbound cells), and the biofilms were scraped using a sterile tip. The serial dilution of supernatant containing the biofilm was made, and all dilutions were plated on a Petri dish containing Mueller Hinton agar. The plates were incubated at 37°C for 24 hours, and the number of colonies was counted. The result was submitted to the normality test (Shapiro-Wilk) and analyzed by analysis of variance (ANOVA) and the Tukey test ($\alpha = 5\%$). Statistical analysis was performed using GraphPad Prism 5 software.

For qualitative analysis, after the period determined to obtain the biofilm, the specimens were carefully washed three times with PBS. The biofilms were then stained with SYTO9 and propidium iodide (PI) using the LIVE/DEAD BacLight Bacterial Viability Kit (ThermoFisher, USA) according to the manufacturer's instructions. Then, the samples were transferred to a confocal plate (SPL, USA) and analyzed by confocal laser scanning microscopy (Carl Zeiss 800 LSM Airyscan, Zeiss, Germany).

3. Results

3.1. Surface characterization

The surface morphology of the samples was investigated using scanning electron microscopy (SEM) and atomic force microscopy (AFM) analysis. In Figure 1, it is possible to observe SEM images of the surface of the Ti25Ta25Nb3Sn alloy after grinding (Figure 1a) and after being subjected to 0.5M (Figure 1b), 1.0M (Figure 1c), and 1.5M (Figure 1d) NaOH alkaline treatment at 60°C for 24 hours, followed by heat treatment at 300°C. For all samples, a nanoporous network similar to the hierarchical

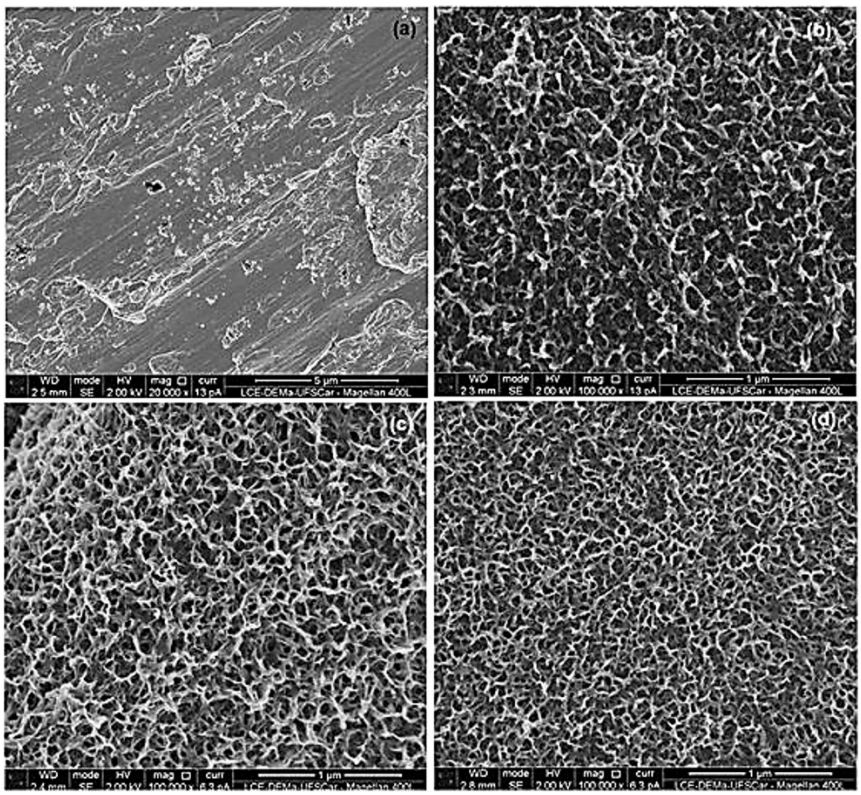

Figure 1. SEM micrographs of the Ti25Ta25Nb3Sn alloy surface: (a) no treated after alkali treatment with (b) 0.5M NaOH (c) 1.0 M NaOH (d) 1.5M NaOH solution at 60 °C and heat treatment at 300 °C.

nanostructured bone tissue was formed without cracks and with interconnected pores. It can be observed that the molarity of the solution affected the size of the nanoporous network structure.

This difference in the topography of the Ti25Ta25Nb3Sn alloy after surface treatment was confirmed in AFM images, and it can be observed in Figure 2. The surface roughness obtained under AFM showed different values of surface roughness between groups. Results showed that for 0.5 M (Ra = 167 nm), roughness was higher than for 1.0 M (Ra = 167 nm) and 1.5 M (Ra = 6.91 nm), and it was altered by the difference in NaOH concentration. It was observed that the higher the NaOH concentration used in the surface treatment, the lower the roughness.

Also, this morphology altered the wettability of the surfaces (Figure 3). Before alkaline treatment, the contact angle was 68°. After alkaline treatment, the contact angle was 0°, indicating that the surface exhibited super hydrophilic behavior for all molarities. It is already reported that hydrophilicity could be induced by surface topography[24], but also by surface composition, such as the formation of the anatase phase[25].

XRD spectra (Figure 4) confirmed the presence of amorphous titanium in the layer; i.e., no anatase or rutile phase was detected.

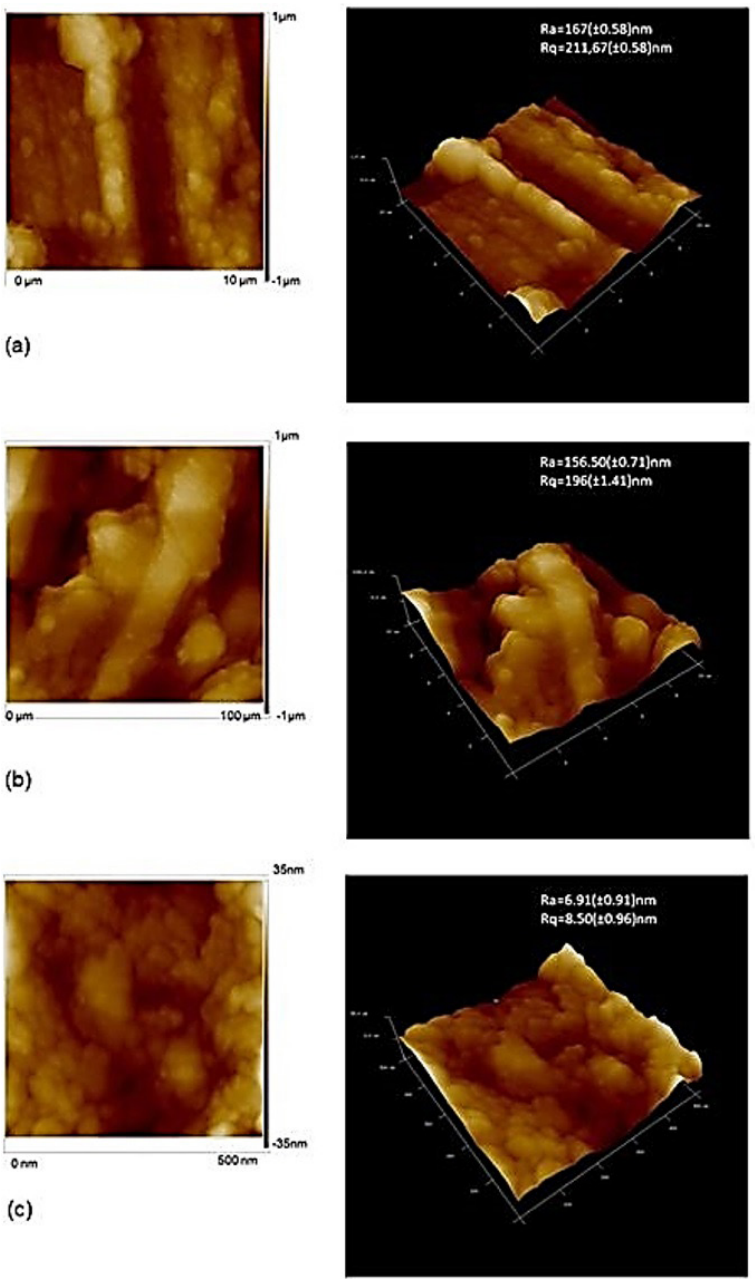

Figure 2. AFM images (2D and 3D surface topography) of the Ti25Ta25Nb3Sn alloy surface after alkaline treatment with different NaOH molarity: (a) 0.5M (b) 1.0M (c) 1.5M.

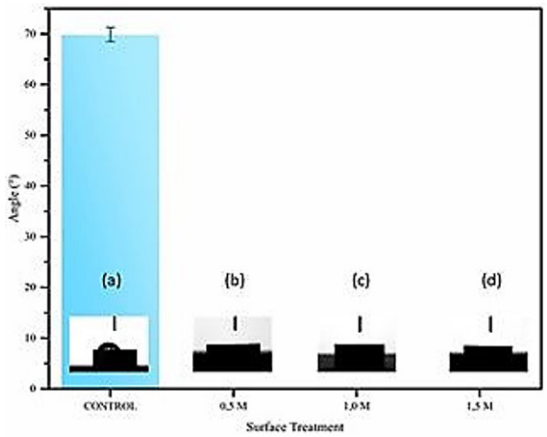

Figure 3. Contact angle measurements and cross-sectional view of a water droplet on the Ti25Ta25Nb3Sn alloy surface (a) no treated and after alkali treatment with (b) 0.5M NaOH (c) 1.0 M NaOH (d) 1.5M NaOH solution and heat treatment at 300°C.

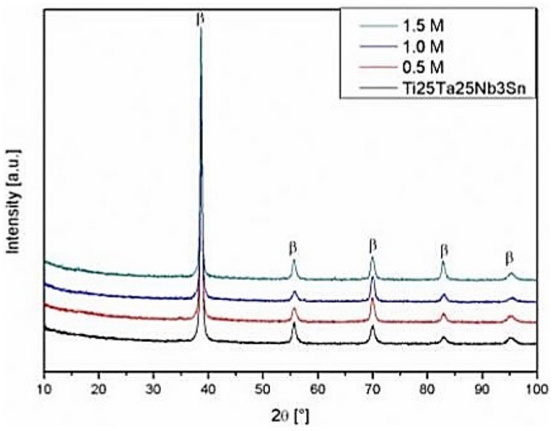

Figure 4. X-ray diffraction of the Ti25Ta25Nb3Sn alloy for different conditions evaluated: no treated and after alkaline treatment (0.5M, 1.0M and 1.5 M NaOH solutions).

3.2. In vitro studies

3.2.1. Cell proliferation and adhesion

In Figure 5, representative fluorescence microscopy images of mesenchymal stem cells stained with DAPI on the Ti25Ta25Nb3Sn alloy after surface treatment with different NaOH solutions (0.5M, 1.0M, and 1.5M) are shown. As shown in Figure 5a and 5b, for the 0.5M group, there are a lot of cells present after day 4 and day 7. Additionally, the cells are spreading and forming clusters. The same results were observed for group 1.0M on day 4 and day 7. However, a reduced formation of clusters was observed (Figure 5c and 5d).

In Group 1, 5 million cells were spreading after day 7, which was completely different from the other groups. It is interesting to observe that the presence of the cluster is important because it indicates interaction between cells. Therefore, for this test, both the 0.5 M and 1.5 M groups exhibit better results. The proliferation of mesenchymal stem cells on treated surfaces was quantified by the Alamar Blue viability assay (Figure 5g). Results showed that treatments did not have a significant effect on the proliferation of osteoblasts. The quantification of nuclei by way of ImageJ indicated that all the surfaces promoted cellular adhesion and growth.

3.2.2. Bacterial adhesion

The analysis of Log10 values (UFC/mL) did not present a statistical difference between the evaluated groups when the S. aureus microorganism was kept in contact with the specimens for 48 hours ($p>0.05$) (Figure 6).

However, in confocal laser microscopy images (Figure 7), it was possible to note that the qualitative data do not corroborate with the quantitative data (CFU/mL). This is because differences in the number and viability of microorganisms can be observed when comparing the experimental groups with the control group (Ti25Ta25NbSn alloy not treated). In specimens treated with 0.5 and 1 M, it is possible to observe a reduction in the number of S. aureus cells when compared to the control. In addition, in the groups treated with 1 and 1.5 M, it is possible to notice a larger number of dead cells (stained in red with Propidium Iodide).

4. Discussion

In this study, alkaline treatment using different NaOH concentrations was used to modify the surface of the new experimental Ti25Ta25Nb3S alloy to obtain a bioactive surface. Alkaline treatment is a chemical treatment used to create a porous layer on titanium and its alloys[26,27]. It was established by the Kokubo group[28] in the 90s for CP titanium and has been studied for many applications.

SEM images showed a morphology similar to a hierarchical nanostructure of bone tissues for all surfaces. This kind of surface, which mimics bone, enhances osseointegration due to surface characteristics such as topography and wettability. According to Gittens et al.[29], the surface topography can also have a direct effect on the biological response of bone. Itis the area that supports new bone formation, which contains a high degree of structural complexity generated after bone resorption, including features at the micro-, submicron-, and nanoscales. Thus, surfaces that mimic bone's hierarchical structure by incorporating some type of surface modification at the microscale or, more recently, a combination of micro- and nanoscale surfaces are very interesting for biomedical applications.

Three different NaOH concentrations (0.5 M, 1.0 M, and 1.5 M) were used. According to AFM results, a sharp conical protrusion was confirmed for 0.5M and 1.0M, while an amorphous and homogeneous nanotextured surface was obtained when used at 1.5M at 60°C for 24 hours and after heat treatment at 300°C.

Another important surface property that directly affects the biological response is surface energy, which is indirectly measured by the liquid-solid contact angle (CA) and is thus related to wettability. Studies showed that alkaline treatment changes the wettability of the surfaces, increasing the hydrophilicity, which influences cell adhesion and

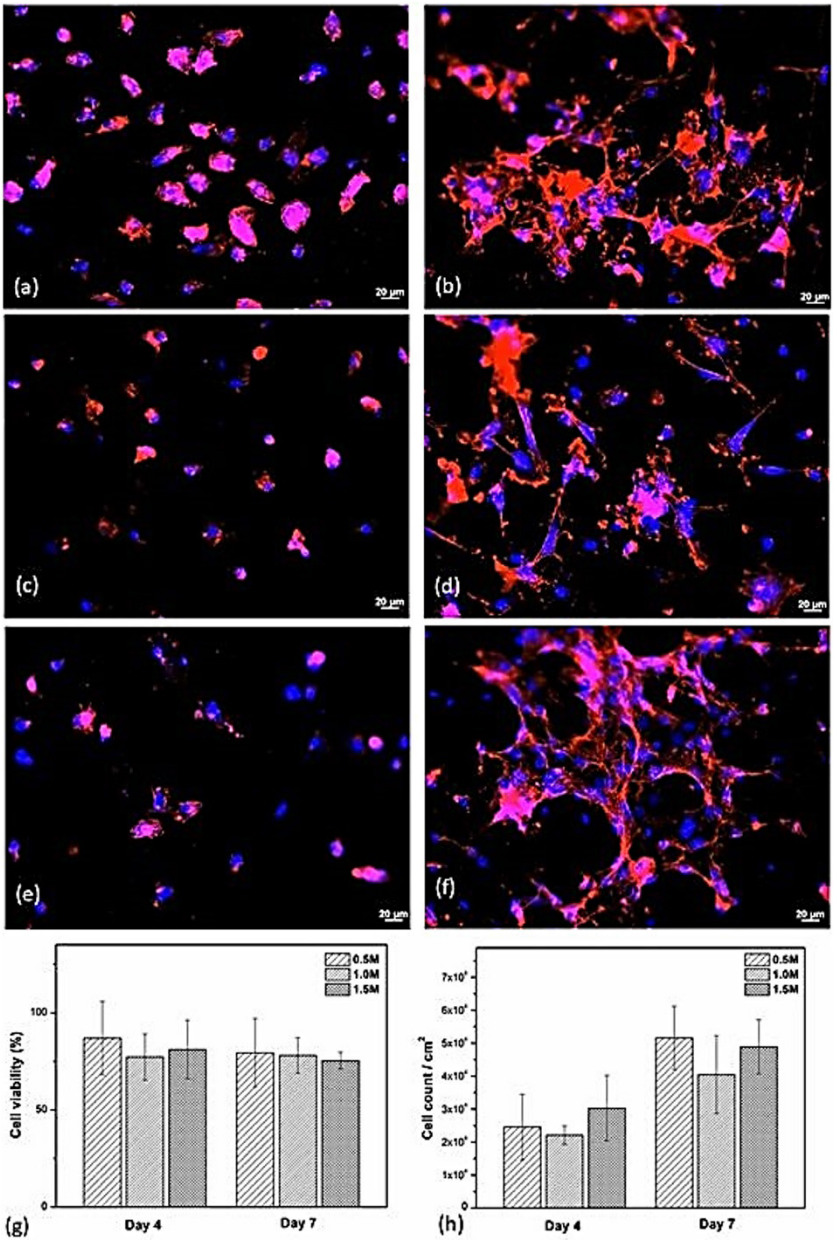

Figure 5. Representative fluorescence microscopy images of osteoblasts cells stained with Dapi on Ti25Ta25Nb3Sn surfaces after alkaline treatment: (a) (b) NaOH 0.5M (c) (d) NaOH 1.0 M (e)(f) 1.5M solution at 60 °C and heat treatment at 300°C for day 4 and day 7, respectively.

spreading[27,30]. The measurements were applied to evaluate the hydrophilic properties of the Ti25Ta25Nb3Sn before and after NaOH alkaline treatment. Despite the different roughness values obtained, all surfaces exhibited superhydrophilic behavior after surface treatment, independent of the molarity used. This led us to conclude that roughness was mandatory in cell response.

The formation of the nanotextured surface was confirmed with AFM analysis, and it was obtained when a 1.5M solution was used in alkaline treatment. The roughness value was 6.91 nm, and according to Anselme et al.[31], osteoblasts were able to differentiate between roughness values ranging from 0.5 to 13 nm, with a rate of proliferation

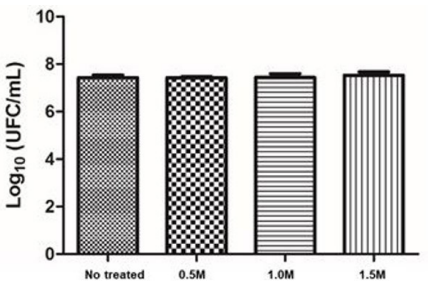

Figure 6. Mean values of Log10 (CFU / mL) of S. aureus biofilms under Ti25Ta25Nb3Sn alloy surface submitted to alkaline treatment with different molarities.

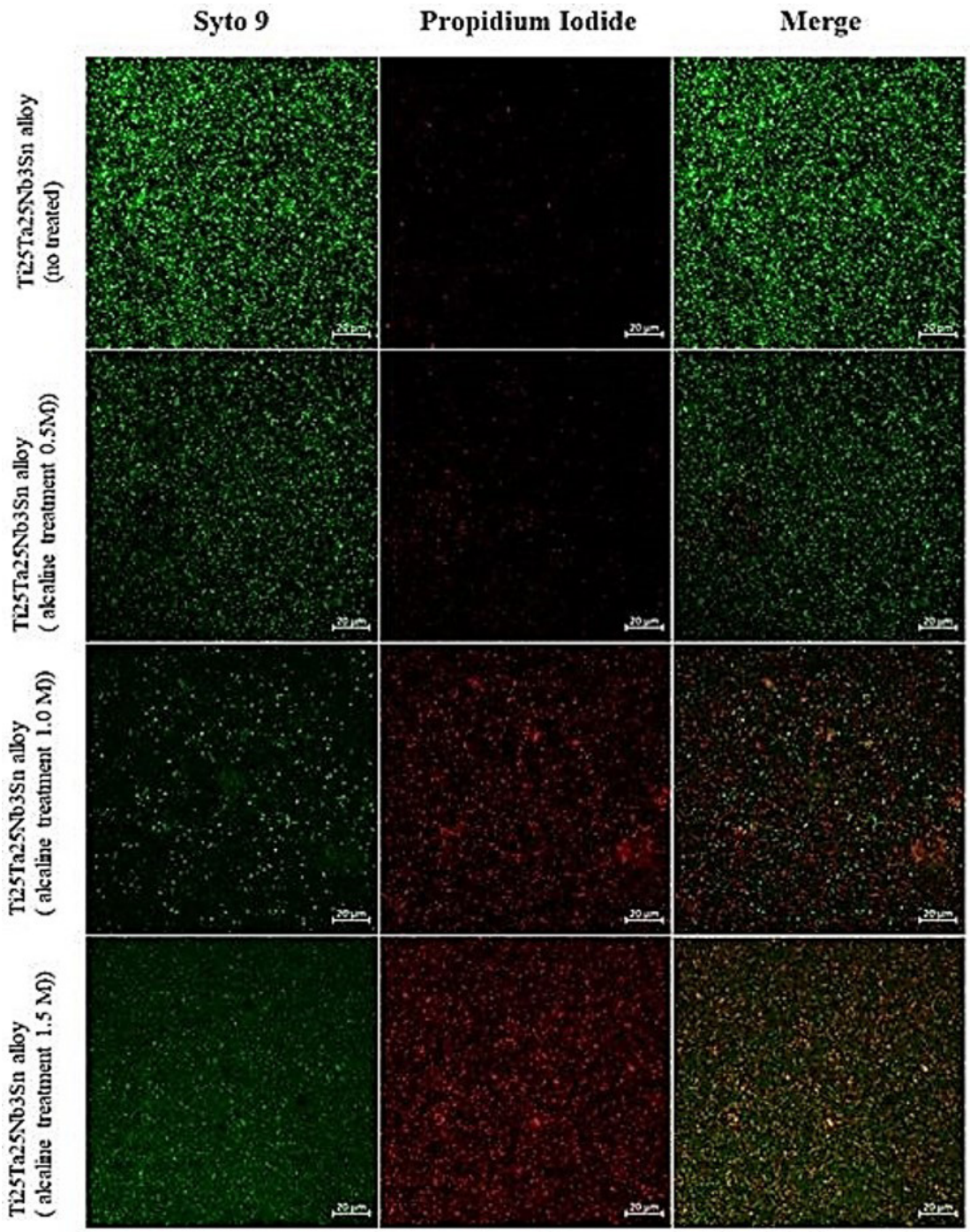

Figure 7. Live/dead fluorescence images of *Staphylococcus aureus* cultured for 48h on the Ti25Ta25Nb3Sn alloy surfaces.

decreasing linearly with increasing roughness. The influence of nanotextured surfaces on cell adhesion was confirmed in fluorescence images (Figure 7). Studies showed that cells attach to different sites on randomly textured surfaces, while nanotextured surfaces provide adsorption of proteins with a link between nanostructure and cells[32]. This behavior is visible in the fluorescence images. In Day 1, cells exhibited a round shape for all treatments. However, after 7 days, their shape became elongated and better spread with filopodia extensions on the nanotextured surface.

Likewise, this kind of surface influenced bacterial adhesion. In confocal laser microscopy images (Figure 7), it was possible to note the difference in the number and viability of microorganisms when comparing the experimental groups with the control group (Ti25Ta25NbSn alloy not treated). In the groups treated with 1 and 1.5 M, it is possible to notice a

larger number of dead cells (stained in red with Propidium Iodide).

Puckett et al.[33] showed in their study that nano-rough Ti surfaces are the best surfaces for inhibiting bacterial adhesion. When compared with conventional surfaces, nanostructured materials exhibit excellent biocompatibility properties due to enhanced protein interaction (including adsorption and conformation), resulting in improved cellular adhesion and tissue growth. The authors demonstrated that there is a linear relationship between nanoroughness, surface energy, and protein adsorption. A surface that has more nanorough features possesses increased surface energy, which leads to greater protein adsorption, resulting in decreased bacteria attachment.

Thus, the alkaline treatment with a 1.0 M solution was more efficient. Because it yielded the best results for cell adhesion and proliferation, as well as a reduction in bacterial adhesion.

5. Conclusions

In this study, the surface of Ti25Ta25Nb3Sn alloy was modified using alkaline treatment. An interconnected layer was formed, and in vitro studies demonstrated the decreased adhesion of S. aureus on the nanoporous surface of Ti25Ta25Nb3Sn after alkaline treatment (1.5M). Likewise, good cell adhesion and proliferation were observed. The development of a material with excellent bulk and surface properties, associated with the reduction of bacterial adhesion, may be feasible for the preparation of dental implants. This could promote the reduction of infections and improve osseointegration.

6. Acknowledgments

We gratefully acknowledge the financial support CNPq – National Council for Scientific and Technological Development (486352-2013-7 and 309469/2015-5) and FAPESP.

7. References

1. Guo Y, Georgarakis K, Yokoyama Y, Yavari AR. On the mechanical properties of TiNb based alloys. J Alloys Compd. 2013;571:25-30.
2. Hacisalihoglu I, Samancioglu A, Yildiz F, Purcek G, Alsaran A. Tribocorrosion properties of different type titanium alloys in simulated body fluid. Wear. 2015;332:679-86.
3. Guo S, Meng Q, Zhao X, Wei Q, Xu H. Design and fabrication of a metastable β-type titanium alloy with ultralow elastic modulus and high strength. Sci Rep. 2015;5:14688.
4. Hussein AH, Gepreel MA, Gouda MK, Hefnawy AM, Kandil SH. Biocompatibility of new Ti–Nb–Ta base alloys. Mater Sci Eng C. 2016;61:574-8.
5. Niinomi M, Liu Y, Nakai M, Liu H, Li H. Biomedical titanium alloys with Young's moduli close to that of cortical bone. Regen Biomater. 2016;3:173-85.
6. Kopova I, Stráský J, Harcuba P, Landa M, Janeček M, Bačákova L. Newly developed Ti–Nb–Zr–Ta–Si–Fe biomedical beta titanium alloys with increased strength and enhanced biocompatibility. Mater Sci Eng C. 2016;60:230-8.
7. Laboulais JN, Mata AA, Borrás VA, Muñoz AI. Electrochemical characterization and passivation behaviour of new beta-titanium alloys (Ti35Nb10Ta-xFe). Electrochim Acta. 2017;227:410-8.
8. Seixas MR, Bortolini C Jr, Pereira A Jr, Nakazato RZ, Popat KC, Claro APRA. Development of a new quaternary alloy Ti-25Ta-25Nb-3Sn for biomedical applications. Mater Res Express. 2018;5:025402.
9. Gosavi S, Alla R, Titanium A. Miracle metal in dentistry. Trends Biomater Artif Organs. 2013;27:42-6.
10. Tanigawa H, Asoh H, Ohno T, Kubota M, Ono S. Electrochemical corrosion and bioactivity of titanium–hydroxyapatite composites prepared by spark plasma sintering. Corros Sci. 2013;70:212-20.
11. Smeets R, Stadlinger B, Schwarz F, Beck-Broichsitter B, Jung O, Precht C et al. Impact of dental implant surface modifications on osseointegration. BioMed Res Int. 2016;2016:6285620.
12. Capellato P, Riedel NA, Williams JD, Machado JPB, Popat KC, Claro APRA. Surface modification on Ti-30Ta alloy for biomedical application. Engineering. 2013;5:707-13.
13. Claro AP, Konatu RT, Escada ALA, Nunes MCS, Maurer-Morelli CV, Dias-Netipanyj MF et al. Incorporation of silver nanoparticles on Ti7. 5Mo alloy surface containing TiO_2 nanotubes arrays for promoting antibacterial coating–in vitro and in vivo study. Appl Surf Sci. 2018;455:780-8.
14. Escada AL, Nakazato RZ, Claro AP. Influence of anodization parameters in the TiO_2 nanotubes formation on Ti-7.5 Mo alloy surface for biomedical application. Mater Res. 2017;20:1282-90.
15. Capellato P, Smith BS, Popat KC, Claro APA. Cellular functionality on nanotubes of Ti-30Ta alloy. Mater Sci Forum. 2015;805:61-4.
16. Kim HM, Miaji F, Kakubo T, Nakamura T. Apatite-forming ability alkali-treated Ti metal in body environment. J Ceram Soc Jpn. 1997;105:111-6.
17. Escada AL, Rodrigues D Jr, Machado JP, Claro AA. Surface characterization of Ti–7.5 Mo alloy modified by biomimetic method. Surf Coat Tech. 2010;205:383-7.
18. Kazek-Kęsik A, Leśniak K, Orzechowska BU, Drab M, Wiśniewska A, Simka W. Alkali treatment of anodized titanium alloys affects cytocompatibility. Metals. 2018;8:29.
19. Rahimipour S, Salahinejad E, Sharifi E, Nosrati H, Tayebi L. Structure, wettability, corrosion and biocompatibility of nitinol treated by alkaline hydrothermal and hydrophobic functionalization for cardiovascular applications. Appl Surf Sci. 2020;506:144657.
20. Escada ALA, Camargo SEA, Vasconcellos LMR, Milhan NVM, Claro APRA. Cytotoxicity analysis of Ti-7.5Mo alloy after biomimetic surface treatment to use as dental material. Mater Res. 2017;20(6):1614-21.
21. Gittens RA, Olivares-Navarrete R, Schwartz Z. Implant osseointegration and the role of microroughness and nanostructures: lessons for spine implants. Acta Biomater. 2014;10:3363-71.
22. Tugulu S, Löwe K, Scharnweber D, Schlottig F. Preparation of superhydrophilic microrough titanium implant surfaces by alkali treatment. Acta Mater. 2010;10:2751-63.
23. Contreras RG, Adachi K, Yokote Y, Sakagami H, Hibino Y, Nakajima H et al. Quantification of enhanced osteoblastic adhesion to ultraviolet-treated titanium plate. In Vivo. 2010;24:519-23.
24. Capellato P, Escada AL, Popat KC, Claro APA. Interaction between mesenchymal stem cells and Ti-30Ta alloy after surface treatment. J Biomed Mater Res A. 2014;102(7):2147-56.
25. Escada ALA, Machado JPB, Schneider SG, Rezende MCRA, Claro APRA. Biomimetic calcium phosphate coating on Ti-7.5 Mo alloy for dental application. J Mater Sci Mater Med. 2011;22(11):2457-65.
26. Camargo WA, Takemoto S, Hoekstra JW, Leeuwenburgh SC, Jansen JA, van den Beucken JJ et al. Effect of surface alkali-based treatment of titanium implants on ability to promote in vitro mineralization and in vivo bone formation. Acta Biomater. 2017;57:511-23.
27. Fujio M, Komasa S, Nishizaki H, Sekino T, Okazaki J. Biocompatibility of titanium surface nanostructures following chemical processing and heat treatment. Front Nanosci Nanotechnol. 2016;3:1-10.

28. Kokubo T, Miyaji F, Kim HM, Nakamura T. Spontaneous formation of bonelike apatite layer on chemically treated titanium metals. J Am Ceram Soc. 1996;79(4):1127-9.
29. Gittens RA, Scheideler L, Rupp F, Hyzy SL, Geis-Gerstorfer J, Schwartz Z et al. A review on the wettability of dental implant surfaces II: biological and clinical aspects. Acta Biomater. 2014;10:2907-18.
30. Hsu SK, Ho WF, Wu SC, Chen YS, Hsu HC. In vitro study of Ti-Nb-Sn alloy surface modified with RGD peptide. Thin Solid Films. 2016;620:139-44.
31. Anselme K, Bigerelle M, Noel B, Iost A, Hardouin P. Effect of grooved titanium substratum on human osteoblastic cell growth. J Biomed Mater Res. 2002;60:529-40.
32. Deligianni DD, Katsala N, Ladas S, Sotiropoulou D, Amedee J, Missirlis YF. Effect of surface roughness of the titanium alloy Ti-6Al-4V on human bone marrow cell response and on protein adsorption. Biomaterials. 2001;22:1241-51.
33. Puckett SD, Taylor E, Raimondo T, Webster TJ. The relationship between the nanostructure of titanium surfaces and bacterial attachment. Biomaterials. 2010;31:706-13.