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Influence of Reactive Sputtering-Deposited Nb_2O_5 Coating On the Ti-6Al-4V Alloy Surfaces: Biomineralization, Antibacterial Activity, and Cell Viability Tests

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Biomedical engineering has been constantly looking for the development of new materials that have bioactive surfaces and that are responsible for new bone formation, induce osteoblast differentiation and present antibacterial properties. Here, we present innovative and applied research to evaluate the influence of niobium pentoxide thin films (Nb_2O_5) deposited on the surface of a Ti-6Al-4V alloy via the reactive sputtering technique on antibacterial activity, on the cellular proliferation of MC3T3 cells and on biomineralization capacity. Results demonstrated the reactive sputtering technique improved the cell viability, the osteogenic performance of cells involved in the osseointegration process, the ability to delay bacterial proliferation within the first six hours of contact with *S. aureus* as well as the capacity to promote rapid amorphous apatite formation *in vitro*. These findings confirm the great potential of the Ti-6Al-4V alloy functionalized with Nb_2O_5 for future applications in implantable devices.

Keywords: *Biomaterials, Orthopedic implants, Titanium alloy, Surface modification, MC3T3 cells*

1. Introduction

Various studies worldwide have focused on enhancing and refining the biofunctional characteristics of metallic materials employed in orthopedic and dental implants. Their objective is to address and mitigate several limitations that hinder their widespread use, including issues related to toxicity, bioinertia, and implant dissolution. As is known, among the range of metallic biomaterials, titanium (Ti) and its alloys have several attractive properties such as good mechanical strength, good resistance to global and localized corrosion and biocompatibility, among others[1-3]. However, as reported in the literature[4], Ti and its alloys are considered inert materials, and although the spontaneously formed titanium dioxide oxide (TiO_2) layer on their surfaces provides a relatively stable interface, it interacts minimally with surrounding tissue and lacks bioactivity to induce bone regeneration, resulting in poor osseointegration. Furthermore, using the Ti-6Al-4V alloy for implants under *in vivo* conditions, such as joints prosthesis, can make the biomaterial susceptible to tribological events and fatigue processes, i.e., the application of load in stationary or dynamic conditions can rupture the oxide film, exposing the material to a global or localized corrosion process when in the presence of aggressive ions[3], such as Cl^- and F^- [5,6].

Thus, in order to improve all these above-mentioned properties, it is essential to develop studies that improve the Ti-6Al-4V alloy surface properties since the surface characteristics of these materials influence the conformational changes of the adsorbed molecules, the adhesion process and the recruitment of tissue-derived, vascular, inflammatory and stromal cells[7].

Studies carried out by this research group have aroused great interest in the functionalization of the Ti-6Al-4V alloy with polycrystalline niobium pentoxide (Nb_2O_5) thin films that are obtained via the reactive sputtering technique[8,9]. The results obtained thus far show that the Nb_2O_5 thin films deposited on the surface of the Ti-6Al-4V alloy act as a protective physical barrier against the corrosion process in a medium containing F^- ions[8]. Consequently, they should prevent the release of aluminum (Al) and vanadium (V) ions that may be related to adverse reactions and that are harmful to the body[10-12].

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In vitro biological experiments use different cells (SaOS-2, human mesenchymal stem cells and osteoblast-like cells) that make it possible to assess the influence of coatings on cell adhesion, proliferation, and differentiation processes[13]. Furthermore, foreign material in the body can stimulate inflammatory and toxic responses, leading to implant instability, tissue necrosis and even rejection[14]. *In vitro* investigations seek to simulate some aspects of cell function and activated signaling after the implantation of a foreign material *in vivo*. Thus, they have a crucial role in the biological evaluation of new biomaterials, allowing for the assessment of several aspects such as cellular interactions with implant materials and implant behavior in a biological environment[13].

Niobium and its oxides have been studied for their ability to improve different biomaterials *in vitro*. Madhavi et al.[15] verified that the implementation of Nb_2O_5 in bioactive glasses improved the bioactivity of these materials, providing antibacterial activity. Studies carried out by Panda et al.[16] showed that the functionalization of the Ti-6Al-4V alloy with niobium-doped hydroxyapatite conferred antibacterial properties against gram-positive and gram-negative bacteria. The incorporation of niobium into amorphous carbon films via the Magnetron Sputtering technique increased cell adhesion and the alkaline phosphatase (ALP) expression of pre-osteoblastic cells grown on their surface[17]. And, finally, the use of niobium has shown bioactivity improvement in grafts in *in vivo* tests, promoting bone formation comparable to that of autogenous bone and without compromising its properties[18].

Considering the various attractive properties of niobium and its oxides, and that there are no studies in the literature obtained by other research groups with similar morphological and structural characteristics[19-21], we present new findings regarding the influence that the Nb_2O_5 thin films deposited via the reactive sputtering technique have on the Ti-6Al-4V alloy's biofunctional properties. Here, this coating's influence on antibacterial activity, MC3T3 cell proliferation and biomineralization capacity was evaluated.

2. Materials and Methods

2.1. Materials

The chemical composition (wt%) of the Ti-6Al-4V alloy is 0.05 N, 0.08 C, 0.015 H, 0.40 Fe, 0.20 O, 5.5-6.75 Al, 3.5-4.5 V, and Ti balance. Ti-6Al-4V alloy specimens were sanded with the aid of silicon carbide (SiC) sandpaper in the granulometries of 800, 1200, 2500 and 4000#. Afterwards, the samples were ultrasonically washed at room temperature with distilled water (10 min) and isopropyl alcohol (10 min). The specimens were coated with Nb_2O_5 films using the reactive sputtering technique. More details regarding the coating technique can be obtained in a previous work published by this research group[8,22-24].

2.2. Differentiation of MC3T3-E1 lineage cells and the evaluation of the influence of the coating on cell proliferation

MC3T3 cells were cultured for a 7-day period in an alpha-minimum essential medium (α-MEM) and supplemented as described by Eurídice et al.[25]. The measurement of the coating's influence on cell proliferation was based on the change in fluorescence after the addition of the resazurin dye[26].

In the physiological pH range, the conversion of resazurin to resorufin is associated with a change in the absorbance spectrum (λ_{max} = 600 nm and λ_{max} = 570 nm, respectively). After the resorufin excitation at the wavelength corresponding to its maximum absorbance, the intensity fluorescence emitted can be recorded (λ_{max}= 590 nm). In contrast, resazurin is weakly fluorescent within the visible spectrum[27]. The cell viability was determined by fluorescence quantification at λ_{em}= 594 nm using a microplate reader (EnSpire, EUA). Cell culture manipulations were performed in a laminar flow hood and incubations in a cell culture incubator with 5% CO_2 at a temperature of 37 ± 1 °C.

2.3. Alkaline phosphatase analysis

After being differentiated into osteoblasts, the cells were also used for alkaline phosphatase (ALP) analysis. The ALP catalyzes the transfer of the phosphate group from the substrate p-nitrophenylphosphate (pNFF) to 2-amino-2-methyl-1-propanol (AMP), forming p-nitrophenol, which has a high absorbance at 405 nm and is proportional to the alkaline phosphatase enzymatic activity of the sample[28]. The cell culture medium was α-MEM containing 1% antibiotics and 10% FBS, and the density was 1×10^5 cells/mL/6-well plate cultured on specimens of Ti-6Al-4V and Ti-6Al-4V/Nb_2O_5 during 7 days at 37 ± 1 °C under 5% CO_2. The culture medium was replaced every 2 days before the collection day. Subsequently, the quantification of ALP activity (Biotécnica, Brazil) was performed in accordance with the instructions set forth by the manufacturer for samples with different stimuli. The analyzes were performed by using a semiautomatic Bioclin 100 analyzer (Quibasa-Bioclin, Brazil) and the results were expressed in mg dL^{-1}.

2.4. Evaluation of antibacterial activity

2.4.1. Agar disk diffusion test

Agar disk diffusion and direct contact tests are the most common to assess the antimicrobial activity of various materials. As reported in the literature[29], the agar diffusion test is strongly influenced by the solubility and diffusibility of the tested agent. The hypothesis that diffusion of some component of the Nb_2O_5 coating could occur, with possible antibacterial activity, was tested. To do so, a volume of 50 mL of *S. aureus* suspension, adjusted to 0.5 on the McFarland scale, was transferred to the blood agar culture medium (see Figure 1a). The samples were placed in the culture with the evaluated surfaces facing downwards as shown in Figure 1b. Then, the plates were incubated for a period of 24 h at a temperature of 37 ± 1 °C (see Figure 1c)[30]. The antibacterial activity was quantified through the inhibition area formed around the tested specimens.

2.4.2. Direct contact antibacterial activity assay

First, an aliquot of bacterial suspension with turbidity corresponding to 0.5 on the McFarland scale was diluted at the proportion of 1:10 in saline solution (0.9% NaCl). Then, 1 µL of this dilution was added together with 200 µL of Brain Heart Infusion Broth on the studied samples' surfaces

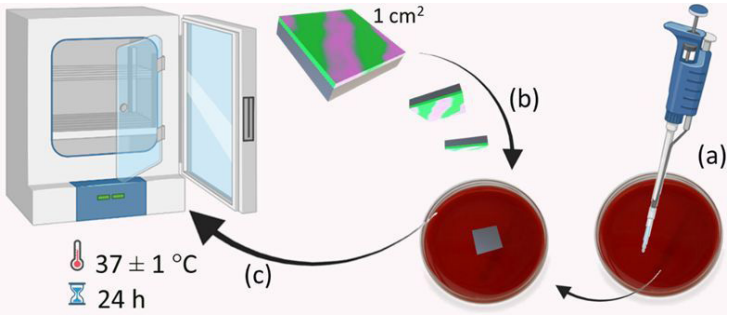

Figure 1. Representative drawing showing the steps for the inhibition halo assay. **(a)** The *S. aureus* suspension, adjusted to 0.5 on the McFarland scale was transferred to the blood agar culture medium, **(b)** the samples were placed in the culture with the evaluated surfaces facing downwards and **(c)** the plates were incubated for a period of 24 h at a temperature of 37 ± 1 °C.

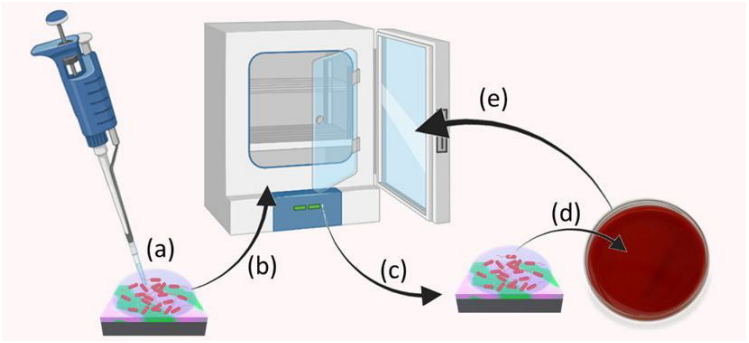

Figure 2. Schematic drawing showing steps for assaying antibacterial activity through direct contact assay. **(a)** bacterial suspension was added together with 200 µL of Brain Heart Infusion Broth on the studied samples' surfaces and **(b)** incubated at a temperature of 37 ± 1 °C. **(c and d)** After 3, 6, 24 and 48 h, 10 µL of this culture were removed and inoculated onto blood agar plates, **(e)** which were then incubated at 37 ± 1 °C for 24 h.

(Figure 2a) and incubated at a temperature of 37 ± 1 °C (Figure 2b). After 3, 6, 24 and 48 h, 10 µL of this culture were removed (Figure 2c and 2d) and inoculated onto blood agar plates, which were then incubated at 37 ± 1 °C for 24 h (Figure 2e). Finally, bacterial counts were performed to verify the coating's influence on bacterial proliferation on the samples' surfaces[30]. The chloramphenicol 100 mg mL^{-1} was used as a positive control.

2.5. Biomineralization test in simulated body fluid

The degree of apatite production in simulated body fluid (SBF) can be used to predict the *in vivo* bone bioactivity level of a material qualitatively and/or quantitatively. This method, which can be used to screen for compounds with bone bioactivity before animal testing, aids in the efficient production of new types of bioactive materials. In the present study, the SBF was prepared according to the recommendations proposed by Kokubo and Takadama[31]. The reagent amounts for the preparation of 1 L of SBF solution and the comparison to the amount of ions found in human blood plasma are presented in Tables 1 and 2, respectively.

The Ti-6Al-4V and Ti-6Al-4V/Nb_2O_5 specimens were submerged in SBF for a 7-day period at a temperature of 37 ± 1 °C. They were then gently washed with ultrapure water and dried at room temperature for 12 h. After this period, they were evaluated via scanning electron microscopy and energy dispersive X-ray spectroscopy (SEM/EDX). The SEM images and EDX maps of the coated and uncoated material were obtained using an analytical electron microscope FEG-SEM JEOL 7001 F equipped with an Oxford EDX light element detector. This all took place at the Federal University of Uberlândia, Department of Chemistry, Minas Gerais, Brazil.

Table 1. Reagents for the preparation of 1 L of SBF.

Reagent	Amount
NaCl	8.035 g
$NaHCO_3$	0.355 g
KCl	0.225 g
$K_2HPO_4 \cdot 3H_2O$	0.231 g
$MgCl_2 \cdot 6H_2O$	0.311 g
HCl (1.0 mol L^{-1})	39 mL
$CaCl_2$	0.292 g
Na_2SO_4	0.072 g
Tris	6.118 g
HCl (1.0 mol L^{-1})	0–5 mL

Adapted from Kokubo and Takadama[31].

Table 2. Nominal ion concentration (mmol L^{-1}) of the SBF compared to human blood plasma.

Ion	Blood plasma	SBF
Na^+	142.0	142.0
K^+	5.0	5.0
Mg^{2+}	1.5	1.5
Ca^{2+}	2.5	2.5
Cl^-	103.0	147.8
HCO^{3-}	27.0	4.2
HPO_4^{2-}	1.0	1.0
SO_4^{2-}	0.5	0.5
pH	7.2–7.4	7.40

Adapted from Kokubo and Takadama[31].

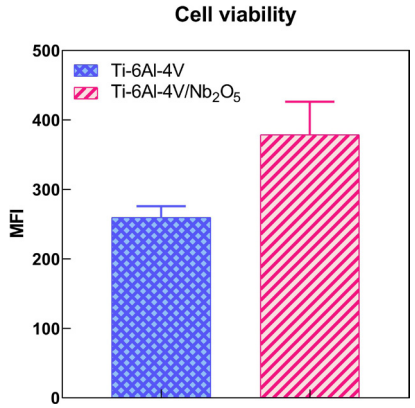

Figure 3. Cell Viability of MC3T3 Cells grown on the evaluated surfaces: Ti-6Al-4V (used as negative control) and Ti-6Al-4V/Nb_2O_5 (given by the Mean Fluorescence Intensity - MFI).

3. Results and Discussion

The titanium alloy Ti-6Al-4V is one of the main materials used for bioimplants. However, it is not bioactive, has no antibacterial activity and can release toxic ions, generating undesirable immune and inflammatory responses[32,33]. The strategy proposed in this research is the application of Nb_2O_5 to biofunctionalize the surface of the Ti-6Al-4V alloy. It is known that oxides in direct contact with the host tissue have a significant effect on the chemical and biological processes that occur at this interface. Furthermore, atomic arrangement and stability, for example, can affect the cell adhesion process, osteoblast proliferation, inflammatory response and biomineralization[33-36]. Figure 3 shows the Nb_2O_5 thin film's influence on the MC3T3-E1 cell viability for a 7-day period. The results clearly show that the Ti-6Al-4V/Nb_2O_5 alloy showed higher cell viability when compared to the base material. These findings only confirm that, in addition to not releasing toxic ions, Nb_2O_5 coatings obtained via sputtering induce greater cell viability of cells involved in the osseointegration process.

An ALP assays was performed to evaluate the osteogenic differentiation of osteoblasts seeds on the samples. As can be seen in Figure 4, the Nb_2O_5 thin films deposited on the Ti-6Al-4V alloy by using reactive sputtering technique promote osteoblast maturation, and consequently osteogenic differentiation. The results obtained in the present work is in line those obtained by Wei et al.[37], i.e., the presence of Nb element increased the ALP activity by 70% as compared to cells planted on hydroxyapatite only. Studies developed by Tan et al.[38], considering *in vivo* and *ex vivo* tests, were used to systematically analyze the osteogenic performance of metallic Nb. And one more time, it was verified the positive influence of Nb element on biological performance[38]. Here, it was demonstrated the osteogenic effect on MC3T3 cells by using ALP analysis, which demonstrated the better performance of the functionalized Ti-6Al-4V alloy containing Nb_2O_5 thin films when compared to the base material.

In the present study, two methods were used (halo inhibition and direct contact) in order to verify the antibacterial effect of Nb_2O_5 thin films. Such an effect was not observed in the agar diffusion assay, as shown in Figure 5. This observation is

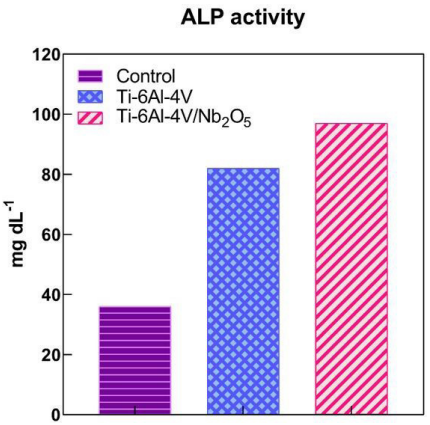

Figure 4. ALP analysis of MC3T3 Cells grown on plate (control), uncoated Ti-6Al-4V and Ti-6Al-4V/Nb_2O_5 for 7 d.

probably due to the absence or impossibility of niobium ion diffusion through the culture plates. Thus, it can be inferred that the deposited films do not have agents with antibacterial capacity with diffusibility in this culture medium. It is worth mentioning that a similar result was also observed in titanium-copper alloy, whose antibacterial activity was verified by applying other methods. More information can be found in the Zhang et al.[39].

On the other hand, through direct contact assays (see Figures 6 and 7), it was possible to observe a negative effect on bacterial growth on the coated Ti-6Al-4V alloy surface at 3 and 6 h, demonstrating the ability to delay the bacterial proliferation within the first six hours of contact with the infecting microorganism. In fact, as reported in the literature[15,40], niobium pentoxide has antibacterial properties. Madhavi et al.[15] reported the antibacterial effect of bioactive glasses implemented with Nb_2O_5, attributing it to the fact that Nb^{5+} ions hinder the growth of bacteria by forming a hyperosmotic atmosphere due to its great ionic strength. In addition, it is also reasonable to assume that the specific charge of Nb and its different oxides provide surface characteristics that can be hostile to some infecting

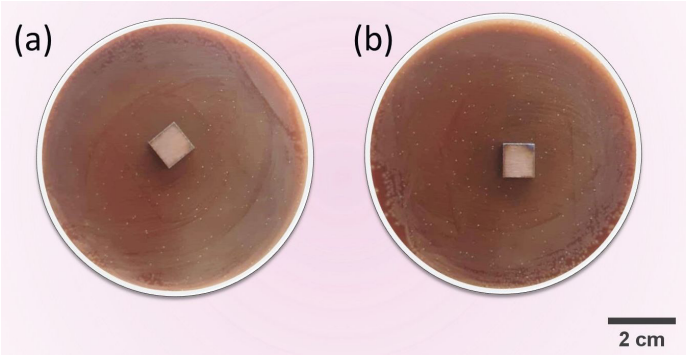

Figure 5. Plates with bacterial culture for 24 h in contact **(a)** with the uncoated Ti-6Al-4V alloy surface and **(b)** in contact with the coated Ti-6Al-4V alloy surface.

microorganisms. This effect on adhesion may also be associated with the coating's crystallinity[41].

It is hypothesized that the observed bacterial fatality rate may be related to the formation of reactive oxygen species (ROS) and interference with 16S rRNA gene replication[42]. And while the exact mechanism of action is undetermined, the results of antibacterial activity tests are quite promising as bacteria from the genus Staphylococcus cause up to two-thirds of orthopedic implant infections[43], typically the most common cause of surgical site infections in patients undergoing hip and knee arthroplasty[44].

In bone tissue engineering, it is desirable that biomaterials have a bioactive surface that promotes the formation of new bone (osteoconductivity), as well as the ability to induce osteoblastic differentiation (osteoinductivity). Simulated body fluid immersion tests have been successfully used to predict the relative performance of biomaterials *in vivo*. The SBF immersion test has the ability to classify various alternative biomaterials quickly and cheaply[45]. The success of this type of assay can be verified in other studies[46-51], being considered the most appropriate *in vitro* method to investigate the bioactivity of materials whose aim is to promote bone bonding and regeneration[49].

The SEM/EDX analysis of the surface of the uncoated Ti-6Al-4V titanium alloy is shown in Figure 8. The Figure 8a presents a micrograph acquired using SE mode where a small area of deposited mineral is indicated. For more details, Figure 8b presents the region displayed in (a) and Figure (c) a micrograph obtained via Backscattered electrons (BSE) with composition details in a local with mineral deposits. Figure 8d exhibits a SEM image obtained by using SE mode, whilst Figures 8e and 8f show the EDX spectra of two regions (highlighted in Figure 8d) of the analyzed sample's surface. The analysis of the obtained spectra (Figures 8e and 8f) demonstrates a small deposition of calcium and phosphorus.

SEM/EDX results showed that the Ti-6Al-4V/Nb_2O_5 surface (Figure 9) displays a higher bioactivity when compared to the uncoated Ti-6Al-4V alloy surface (Figure 9). The SEM images displayed in Figures 9a and 9b were obtained by using SE and 9 (c) BSE modes. A large area of mineral deposits with different compositional characteristics can be seen in the Nb_2O_5 coating. Figure 9f presents an image, obtained via SE, with more details on the morphology of these findings. Figures 9g and 9h, obtained via EDX, confirm calcium

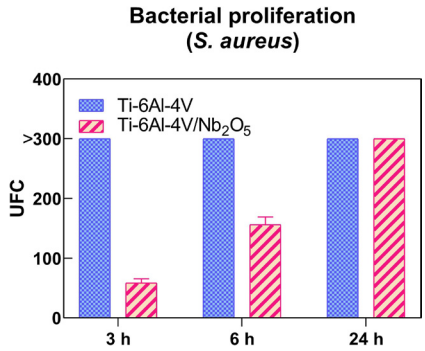

Figure 6. Count of Colony Forming Units (CFU) of *S. aureus* in direct contact with the Ti-6Al-4V alloy surface (used as negative control) and in contact with the coated Ti-6Al-4V/Nb_2O_5 alloy surface. In the positive control there was no bacterial proliferation, therefore the results are not shown.

and phosphorus accumulations on the Nb_2O_5 coating in the analyzed area. The semiquantitative analysis of the obtained spectra (Figures 9d and 9e) demonstrated a deposition of calcium and phosphorus superior to the spectra obtained for the uncoated material (Figures 8e and 8f). These results are an indicative of the rapid amorphous apatite formation with a Ca/P ratio close to 1.5 in only 7 days of immersion in SBF. The calcium distribution throughout the Ti-6Al-4V/Nb_2O_5 sample corroborates the proposal that the heterogeneous nucleation mechanism of hydroxyapatite starts with Ca^{2+} ions[50].

Figure 10 illustrates a simplified schematic model proposed to elucidate the mechanism of hydroxyapatite nucleation on the Ti-6Al-4V surface containing Nb_2O_5 thin films, based on the results obtained in the biomineralization assays. As can be seen in Figure 10a, the Ti-6Al-4V/Nb_2O_5 alloy surface immersed in SBF becomes negatively charged and attracts positively charged Ca^{2+} ions (Figure 10b). Subsequently, phosphate ions that are negatively charged combine with calcium ions (Figure 10c and 10d) and in a physiological environment convert to amorphous apatite with a Ca/P ratio lower than 1.5 (Figure 10e). In the following steps, at a ratio close to 1.65, stable hydroxyapatite is formed (Figure 10f)[52]. In Figure 10g it is possible to verify the format of the unit cell. This formed mineral layer has a similar composition to bone. In *in vivo* tests, an improvement in bone deposition on

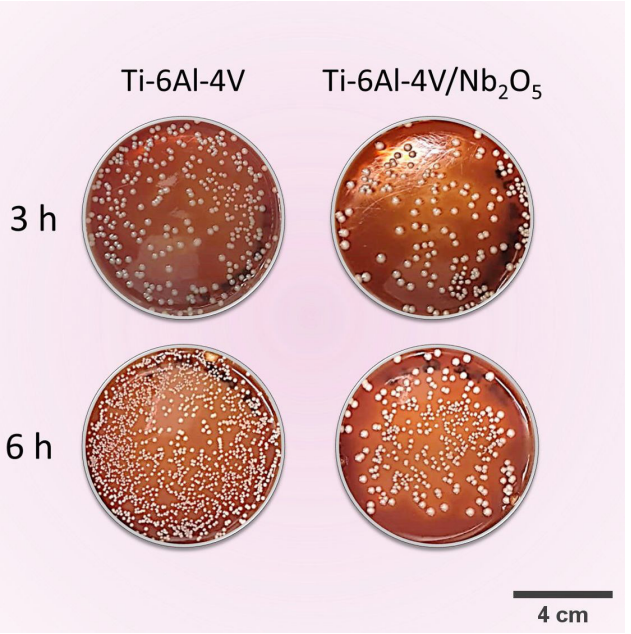

Figure 7. Qualitative result of *S. aureus* incubated at 37 ± 1 °C for 24 h after direct contact for 3 and 6 h with the Ti-6Al-4V alloy surface and in contact with the coated Ti-6Al-4V/Nb_2O_5 alloy surface. In the positive control there was no bacterial proliferation, therefore the results are not shown.

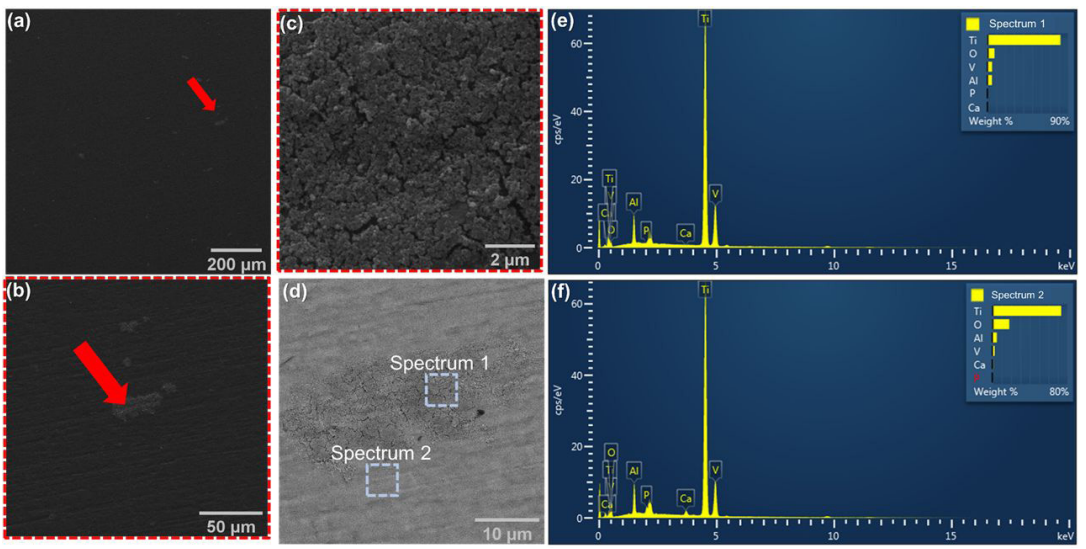

Figure 8. Analysis via SEM/EDX of the uncoated Ti-6Al-4V titanium alloy surface. **(a)** micrograph acquired using SE mode, **(b)** the region indicated in **(a)** at a higher magnification, **(c)** micrograph obtained via BSE of the region indicated in **(b)**, **(d)** micrograph obtained via SE, and **(e, f)** show a spectra of two regions highlighted in **(d)**.

osteoconductive surfaces is observed, leading to a stronger union at the bone/implant interface[45].

For the first time, it was evaluated the influence of polycrystalline Nb_2O_5 film coatings, which have unique structural and morphological characteristics, on the viability and proliferation of osteoblasts, and on bone bioactivity. It also explored the coating's influence on the ability to inhibit the growth of bacteria cultured on the Ti-6Al-4V alloy surface. The results indicate improved bioactivity in all tests performed. This coating provided antibacterial activity against *S. aureus*, better cellular response in the viability and proliferation of MC3T3 osteoblasts and increased apatite formation on the modified surface, confirming the potential for future evaluations of this coating in *in vivo* tests.

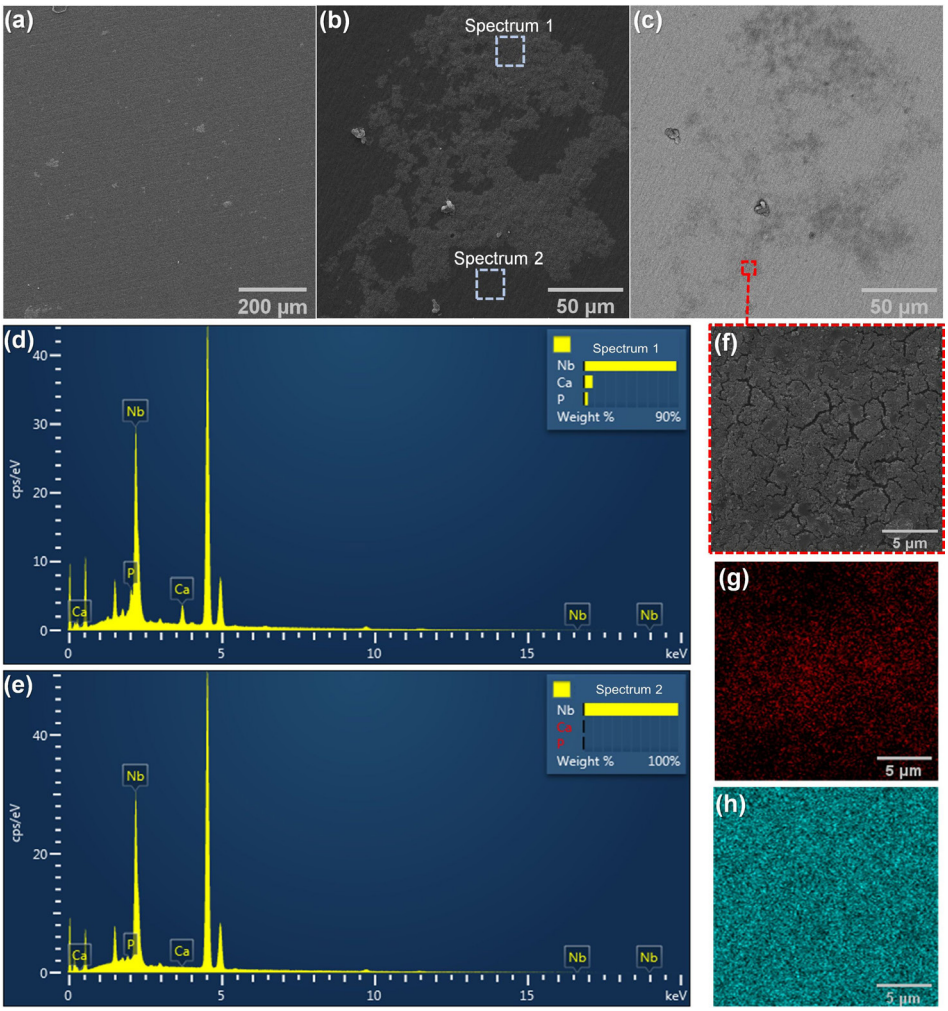

Figure 9. Analysis via SEM/EDX of the Ti-6Al-4V/Nb_2O_5 titanium alloy surface. **(a, b)** micrograph obtained via SE and **(c)** BSE mode, **(d, e)** spectra of two regions of the analyzed sample's surface. **(f)** region highlighted in **(c)** obtained via SE mode at a higher magnification, **(g, h)** maps obtained via EDX to assess the presence of Ca and P, respectively.

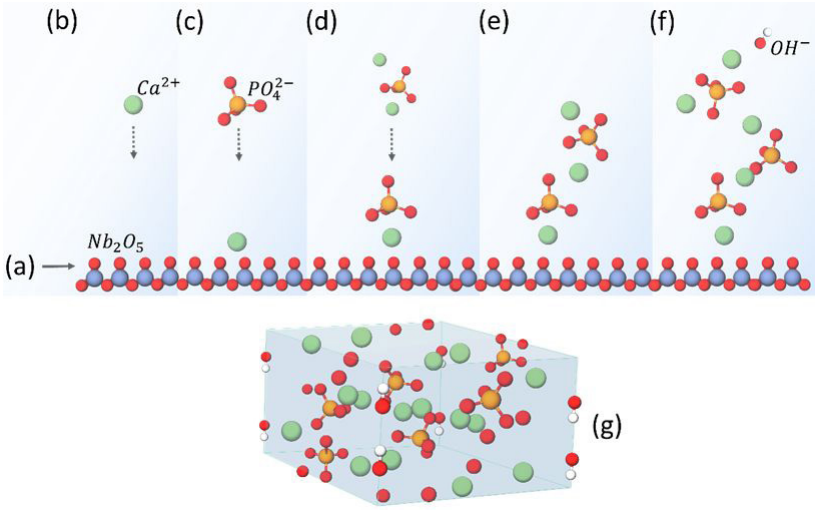

Figure 10. Simplified schematic model proposed to understand the nucleation mechanism of hydroxyapatite on the Ti-6Al-4V alloy surface containing Nb_2O_5 thin films.

4. Conclusions

In this study, we revealed new insights pertaining to the impact of Nb_2O_5 thin films deposited by using the reactive sputtering technique on the biofunctional properties of the Ti-6Al-4V alloy. The evaluation specifically encompasses the coating's effects on antibacterial activity, MC3T3 cell proliferation as well as biomineralization capacity. The results obtained in the present work are innovative and applied, placing the Ti-6Al-4V alloy containing Nb_2O_5 coatings as an attractive material in the biomedical sector. It can be concluded that the functionalized material, when compared to the base alloy, presents greater cell viability, the osteogenic performance of cells involved in the osseointegration process, ability to delay bacterial proliferation in the first six hours of contact with the infectious microorganism as well as the ability to promote rapid formation of amorphous apatite *in vitro*, predicting bone bioactivity *in vivo*. All these findings support the hypothesis that the Nb_2O_5- based coating confers relevant bioactive properties for the viability, adsorption and proliferation of cells cultured on the surface of the Ti-6Al-4V alloy.

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