Titanium Biomimetically Coated With Hydroxyapatite, Silver Nitrate and Polycaprolactone, for Use In Biomaterials (Biomedicine)

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Titanium is used in orthopedic and orthodontic implants because it has good corrosion resistance and excellent biocompatibility. Thus, studies seek to obtain a coating to improve the adhesion between the bone and the implant, by modifying the metal’s surface. The objective of this work was to biomimetically coat C.P. Ti with hydroxyapatite doped with silver nitrate, a component with antimicrobial properties, coating the metallic-ceramic composite with a polycaprolactone polymer film, which is known by generate improved implant-tissue interaction, and reducing postoperative complications from bacterial infections. The characterization of the material demonstrated the existence of the coating overall surface of the metallic substrate. The results obtained from the bacterial culture tests with Staphylococcus aureus showed that nitrate was effective in reducing the amount of live bacteria present in the supernatant, as well as those adhered to the surface of the material. In addition, the polymeric coating did not prevent the release of the bactericidal agent, not interfering in the effect there.

Keywords: Titanium, hydroxyapatite, polycaprolactone, biomimetic coating.

1. Introduction

Alterations in the functioning of cells, tissues or organs (degenerative diseases), inflammations affect millions of people around the world. With this motivation, the search for new technologies that aim to improve and optimize products and processes is becoming progressively constant. The search for materials that allow the substitution of tissue and bones has increased significantly in the last few years1-4. Studies on the application of biomaterials of natural or synthetic origin have been gaining more space in biomedicine, as they allow the substitution or improvement of tissues, organs and body functions5-6.

The search for functional materials has increased significantly in the last few years, and commercially pure titanium (C.P. Ti) and its alloys have broad application in this area because of their high degree of resistance to corrosion in bodily fluids, as well as their low modulus of elasticity and characteristics as biocompatible materials4,7-9.

With the intention of promoting and accelerating osseointegration, reducing possible post-surgical damage and, consequently, accelerating patient recovery, a coating of hydroxyapatite (HA), the principal component of bones, is necessary on the metallic implant. This coating facilitates the chemical bonding between the live tissue and the implanted material, as it is a bioactive substance10,11.

As a surgical procedure, some bacterial infections can occur, resulting in recurring surgical interventions on the patient. In this sense, studies are being developed for the application of bactericidal agents on the surfaces of the materials to be implanted. Silver nitrate (AgNO3) has been used for this purpose, since silver can cause the disruption of bacterial cell membranes12. Polycaprolactone (PCL) is a synthetic polymeric material, known by its biocompatibility and biodegradability, used also as a biological agent. In addition, PCL has the capacity to form films on metallic and ceramic surfaces13,14.

Despite all caution, surgical procedures for implants involve risks of infection associated with the contamination of the implant itself and/or the conditions of the patient and the surgery. Thus, bacterial infection and the formation of biofilms are the main causes of post-surgical complications regarding implants, followed by rejection reactions. The formation of a biofilm corresponds to the bacterial adhesion on a surface which can be of biotic (animal or plant tissue) or abiotic (metals, polymers) origin. This is considered a factor in 80% of the cases of infections associated with biomedical implants. As such, an analysis of this parameter in necessary during the development of a new biomaterial15.

In the literature, studies conducted by Smith show that PCL coatings on artificial titanium prostheses are possible, since the material presents good adhesion to the metallic matrix16.

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Shoja and collaborators demonstrated in their studies that silver nitrate has antimicrobial properties which also guarantees the controlled release of silver ions (Ag⁺)\(^3\). The effect of corrosion on the implants is another determining factor in the choice of the material that will be applied, since the release of ions in the body can be very harmful to the health of the implanted patient. In addition, corrosion may cause problems such as excessive wear of the implant, requiring replacement before the scheduled time, besides the annulment of implant efficiency resulting in loss of implant material\(^7\).

The objective of this work was the study of the attainment and characterization of C.P. Ti (grade 4) coated with hydroxyapatite doped with silver nitrate and later coated with PCL, for potential use in implants. And, afterwards, the evaluation of the bacterial viability, the formation of biofilms and the corrosion behavior in an electrolytic solution of Simulated Body Fluid (SBF).

2. Materials and Methods

Commercially pure titanium (C.P. Ti) discs, grade 4, with chemical composition according to ASTM F67-06\(^6\) was used as a metallic substrate for study. In total, ten samples were used for each coating condition: one sample for characterization (Scanning Electron Microscopy (SEM), Energy Dispersive X-ray (EDX), X-ray Diffractometry (XRD) and Fourier Transform Infrared (FTIR)), three samples for corrosion tests, three samples to evaluate bacterial viability and three samples for formation of biofilms.

For surface preparation, C.P. Ti discs were subjected to 180 mesh wet-grounded sandpaper, followed by an ultrasonic bath with detergent, distilled water and isopropyl alcohol. Subsequently, the samples were immersed in a 5 M NaOH solution and placed in an oven at 60 °C for 24 hours, a step defined as alkaline treatment. Posteriorly, the discs were submitted to thermal treatment at 600 °C for 1 hour, and cooled to room temperature.

The coating with hydroxyapatite was performed by the biomimetic method, which occurs in two stages: nucleation and growth. In the first step, alkaline-treated C.P. Ti discs were immersed in a 0.002 M solution of sodium silicate (pH = 7.4) for seven days in an oven at 36.5 °C. In the second step, the samples were immersed in SBF solution, with a concentration of 1.5%, for seven days in an oven at 36.5 °C, changing the solution each 48 hours, to ensure the supersaturation of the environment, favoring the formation of the hydroxyapatite layer. Subsequently, the samples were washed with deionized water, dried at room temperature and left in a desiccator.

After biomimetic coating, samples were submerged in 50 mL of a solution of 10 ppm of AgNO\(_3\) and kept in an oven at 37 °C for 48 hours, then dried to room temperature in a desiccator. These samples were named C.P. Ti+HA+AgNO\(_3\).

For the polymeric coating, PCL (MM 70000-90000, density 1145 g/mL at 25 °C, Sigma Aldrich, USA) was used. The solution was prepared from the dissolution of PCL in chloroform in the ratio 1:100 (m/v). C.P. Ti samples, coated with HA and doped with AgNO\(_3\), were immersed in the polymeric solution for 10 seconds, and then dried at room temperature in a desiccator. These samples were named C.P. Ti+HA+AgNO\(_3\)+PCL.

Scanning Electron Microscopy micrographs of the samples were obtained in a FEI microscope, model Inspect 550, coupled to an EDX detector of the brand EDAX, model Apollo X. X-ray diffraction analysis (XRD) was performed in a Rigaku Diffractometer, model Ultima IV, with Cu K\(\alpha\) radiation. The samples were analyzed in a 20 range from 10º to 90º, with step of 2º per minute.

Fourier Transform Infrared analysis was performed using an Attenuated Total Reflection (ATR) accessory, in a Perkin Elmer, model Spectrum 100 FTIR Spectrometer. The samples were analyzed from 500 to 4000 cm\(^{-1}\) in order to identify the characteristic functional groups present.

The corrosion tests were carried in triplicate using three samples for each coating condition out by the potentiodynamic polarization technique, and were performed using an electrochemical cell, working electrode, platinum counter electrode and the saturated calomel reference electrode (SCE). The electrolytic solution was 1.5 SBF at pH 7.4 and 36.5 °C. The polarization curves were obtained by a continuous scanning rate of 1 mV.s\(^{-1}\).

The bacterial viability tests and biofilm formation were performed in triplicate (using three samples for each coating condition). A bacterial culture test was performed using Staphylococcus aureus, with polyethylene (PE) plate as the control substrate. The titanium samples were placed on a 24-well Elisa plate, with the addition of 1000 µL of Luria-Bertani medium in each well along with 5 µL of the bacterial culture. The samples were incubated for 24 hours, and after this period, the supernatant was removed and transferred to another 24-well Elisa plate. To verify the amount of bacteria present in the medium, the Spectra Count\(^\text{TM}\)Packard spectrophotometer was used at 570 nm.

The plates were then washed twice with PBS and dried at 65 °C for 1 hour. They were cooled to room temperature and stained with 500 µL of 1% crystal violet for 10 minutes. The dye was removed from the plates, which were then washed twice with PBS and dried overnight. The biofilms formed were dissolved by adding 95% ethanol (500 µL) to each well and shaking for 2 hours. The contents were diluted 20 times and then the absorbance was recorded at 570 nm. For comparative purposes, a C.P. Ti sample coated only with hydroxyapatite (Ti+HA) was included in this bacterial analysis.
3. Results and Discussion

3.1 Microstructural Characterization

Figure 1a shows the micrographs of the Ti+HA+AgNO₃ obtained by SEM, demonstrating that the coating with hydroxyapatite occurred, besides the observation of agglomerates (HA+Ag), heterogeneously distributed on the surface of the samples. Similar results were obtained by Eraković (2013)²⁰ and Ciobanu (2011)²¹.

Figure 1b shows the detail of the agglomerate of HA+Ag. There was the presence of metallic silver (Ag) in a denser network of the hydroxyapatite layer. The hydroxyapatite structure possesses Ca⁺ ions that can be substituted by metallic cations (Ag⁺, Cu²⁺, Zn²⁺) of the bactericides. These substitutions alter characteristics such as texture and crystallinity of the surface layer²²-²⁴.

However, in this work the possibility of Ag⁺ substitution in the HA structure was discarded, since no changes were observed in the crystallographic planes of the HA and Ag, confirmed by the X-ray diffractogram.

According to Kim (1996), Kokubo (1996) and Liu (2013), the treatment of Ti with NaOH promotes a surface alteration of the sample, increasing its bioactivity and favoring HA nucleation, which does not occur in samples that did not undergo alkaline and thermal treatments²⁵.

Figure 1c presents the spectrum obtained by EDX and confirms the presence of the element silver and hydroxyapatite constituents. The results of the EDX semi-quantitative microanalysis at point A, indicates the presence of the chemical elements O, P, Ca and Ag (in weight %): 37.7, 0.4, 0.8 and 5.5, respectively.

Figure 1. SEM micrograph for the sample Ti + HA + AgNO₃ (a, b) and (c) spectrum obtained in the microanalysis of EDX at point A
The micrograph shown in Figure 2 correspond to the samples Ti+HA+AgNO₃+PCL obtained by SEM along with the spectrum obtained by EDX microanalysis in the indicated regions. In Figure 2a, the micrograph showed silver and calcium particles sparsely deposited on the titanium surface, and some particles formed aggregates (region A). To verify the chemical elements present, EDX analyses were performed, as shown in Figures 2b.

The EDX spectrum undertaken in region A (Figure 2b) shows the presence of chemical elements typical of PCL (C and O), as well as Ca and P, the main components of HA, and Ag corresponding to the AgNO₃ used in the doping of the coating. The presence of Na peaks in these spectra is due to the NaOH used in the alkaline treatment.

Micrographical analysis made by Ferreira (2018) allowed the observation of the presence of spherulites in the PCL film. In its EDX spectra, the main chemical elements found in the polymer (carbon and oxygen) were identified. The characteristic peaks of the titanium α-phase can be found in the diffractograms of C.P. Ti, Ti+HA+AgNO₃, Ti+HA+AgNO₃+PCL, this α-phase has a compact hexagonal structure. In addition, the diffractograms of PCL and Ti+HA+AgNO₃+PCL indicate the presence of characteristic peaks of PCL. PCL presents an X-ray pattern with two intense reflection peaks at 2θ = 21° and 2θ = 29°, reported by Wei et al. and Ferreira et al. The presence of HA and Ag was observed in the diffractograms of the samples Ti+HA+AgNO₃ and Ti+HA+AgNO₃+PCL. The distinctive peaks of HA are located at 2θ = 33° (JCPDS 09-0432) as observed by Chen et al. (2006). Diffraction patterns obtained by Rakngarm et al. also showed HA peaks in a range of 2θ = 30-33°. They presented a significantly lower intensity in relation to the α-phase peaks of C.P. Ti.

The peaks detected at 2θ = 37.8°, 43.8°, 64.3°, 77.5° and 81.5° refer to the crystallographic planes of Ag. As noted by Andrade et al. (2014), these correspond to the (1 1 1), (2 0 0), (2 2 0), (3 1 1) and (2 2 2) planes of Ag face-centered cubic lattice (JCPDS 04-0783).

Figure 2. (a) Micrograph obtained by MEV for Ti + HA + AgNO₃ + PCL. (b) Microanalysis obtained by EDX in region A.

Figure 3. X-ray diffraction of C.P. Ti, Ti + HA + AgNO₃ and Ti + HA + AgNO₃ + PCL.
The structure of hydroxyapatite allows easy isomorphic cationic and anionic substitutions. Ca$^{2+}$ can be substituted by metallic cations, such as Pb$^{2+}$, Ag$^{+}$, Cd$^{2+}$, Cu$^{2+}$, Zn$^{2+}$, Sr$^{2+}$, Co$^{2+}$, Fe$^{3+}$. The phosphate groups can be substituted by carbonates and vanadates and the hydroxyls by carbonates, fluorides and chlorides. These substitutions can alter crystallinity, the network parameter, crystal dimensions, surface texture, the stability and solubility of the structure of hydroxyapatite$^{25,29,31}$.

Vieira (2013) observed the presence of the β-TCP phase in C.P. Ti samples with hydroxyapatite coating and doped with 20 and 100 ppm of AgNO$_3$. This phase is a result of the transformation in the hydroxyapatite structure by the substitution of Ca$^{2+}$ ions (from the HA) by Ag$^{+}$ ions (from the AgNO$_3$)$^{23}$. However, Sousa and collaborators (2018) did not observe the presence of the β-TCP when the samples were doped with 10 ppm of AgNO$_3$.$^{32}$ As such, the choice of working with a concentration of 10 ppm of AgNO$_3$ was made.

β-TCP is a phase deficient in Ca$^{2+}$ and one which can foster a structural disorganization in the HA, generating changes in properties such as network parameters, surface texture, crystallinity and material stability. In the samples Ti+HA+AgNO$_3$ and Ti+HA+AgNO$_3$+PCL, it was observed that the presence of the β-TCP phase did not occur.

The use of spectroscopy in infrared allowed the monitoring and evaluation of the apatite coatings formed, with the identification of absorption bands stemming from the phosphate (PO$_4^{3-}$), carbonate (CO$_3^{2-}$) and hydroxyl (OH-) groups$^{32,33}$. Figure 4 presents the results obtained by the FTIR of the C.P. Ti, C Ti+HA+AgNO$_3$, Ti+HA+AgNO$_3$+PCL.

The absorption bands corresponding to the vibration of the phosphate and hydroxyl groups are directly linked to the presence of apatites. In the coated samples (Ti+HA+AgNO$_3$ and Ti+HA+AgNO$_3$+PCL), the band observed in the region between 3670-3570 cm$^{-1}$ refers to the stretching of the OH-group, while the peaks at 1196 and 1022 cm$^{-1}$ indicate the stretching of the phosphate group (PO$_4^{3-}$) and, at 563 cm$^{-1}$, its vibration.

The characterization of the type of hydroxyapatite was undertaken through the analysis of the vibrations of the CO$_3^{2-}$ group. The peaks at 1514 and 880 cm$^{-1}$ in Ti+HA+AgNO$_3$ sample refer, respectively, to the stretching and the vibration of the type A CO$_3^{2-}$, while the peak at 1465 cm$^{-1}$ refers to the stretching of the CO$_3^{2-}$.

The same could be observed in the sample containing polymeric coating, where the peaks observed at 1514 cm$^{-1}$ and 1412 cm$^{-1}$ refer to the stretching of the A and B type groups, respectively, characterizing the AB type apatite$^{25}$. These results corroborate with the observed, confirming the results obtained through the SEM/EDX and XRD of the Ti+HA+AgNO$_3$.

The peaks at 1031 and 955 cm$^{-1}$ refer to the vibrations of the PO$_4^{3-}$ group, while the peak at 567 cm$^{-1}$ refers to the stretching of the PO$_4^{3-}$ group. The vibration of the OH refers to the peak at 3662 cm$^{-1}$. At 1621 cm$^{-1}$, in Ti+HA+AgNO$_3$ sample it refers to the deformation of the H$_2$O group.

The absorption bands in the range of 2862 and 2942 cm$^{-1}$ in Ti+HA+AgNO$_3$+PCL sample derive from to the deformation of CH$_2$ of the PCL, interconnected to the inorganic network. The band at 1726 cm$^{-1}$ is attributed to the stretching vibration of the carbonyl groups (C=O) present in the PCL. Among the characteristic vibrations of the PCL, the absorption bands 1233, 1107 and 1042 cm$^{-1}$ correspond to the asymmetric COC vibration$^{26}$.

3.2 Corrosion Testing

Figure 5 shows the potentiodynamic polarization curves of C.P. Ti; Ti+HA+AgNO$_3$ and Ti+HA+AgNO$_3$+PCL and Table 1 shows the electrochemical parameters obtained from the polarization curves.

It was possible to observe that in the sample C.P. Ti there is a clear passive region, where the current density was practically constant, while the potential values suffer variations. This behavior indicates the formation of a passive film (possibly titanium oxide) on the surface of C.P. Ti (Ipass = 1.116x10$^{-5}$ A/cm$^2$), which breaks at a potential of around 1.35 V. Afterwards, a layer of oxide was once again formed, indicating that the oxide adhered to the surface of the C.P. Ti and was stable there. Nevertheless, this behavior of passive film rupture did not occur in the sample Ti+HA+AgNO$_3$. 
Corrosion potential (Ecorr) of Ti+HA+AgNO₃ (-0.2944 V) is similar to other coated conditions like Ti+PCL₂⁶ (-0.3061 V) and Ti+HA₃² (-0.321 V), but higher than C.P. Ti (-0.4956 V). This indicates that the coated are more stable and that the coatings slow the onset of the corrosive process. However, the corrosion potential of the Ti+HA+AgNO₃ +PCL was the largest (-0.1239 V) in relation to the other samples. This result was also observed by Catauro (2018), and it is due to the fact that the PCL is more inert and stable in an SBF medium when compared to the biomimetic coating (HA)₃⁶. Therefore, the use of PCL may protect the implant against the attack of body fluids and improve its biocompatibility. The sample Ti+HA+AgNO₃ exhibited the largest passive current density (Ipass = 1.12x10⁻³ A/cm²), as a result of the interaction of silver nitrate with hydroxyapatite, which alters the coating structure, reducing the speed of passive film formation. This fact is reinforced by the results obtained by Sousa (2018), in a Ti+HA sample, in a similar medium, which exhibited a lower passive current density (Ipass = 5.472x10⁻⁵ A/cm²)₃².

### 3.3 Bacterial Viability Trials

Aiming to evaluate the bacterial viability and the formation of biofilms on the samples, a bacterial viability trial was conducted with *Staphylococcus aureus*, reported by Gristina and Costerton (1985), in infections associated with biomaterials. The results obtained are shown in Figures 6 and 7. Additionally, an analysis of bacteria in samples of Ti+HA was performed, for comparative purposes.

**Figure 5.** Polarization curves obtained in SBF solution, at 36.5 °C and pH 7.4, for the samples: C.P. Ti, Ti + HA + AgNO₃ and Ti + HA + AgNO₃ + PCL

**Figure 6.** Bacterial viability trial for *Staphylococcus aureus*.

**Figure 7.** Biofilm formation trial for *Staphylococcus aureus*.

Figure 6 shows the bacterial viability in the supernatant after 24 hours of treatment with the samples. Analyzing the values obtained, it is possible to observe that the condition Ti presented a smaller reduction in the number of viable cells (8%), followed by the condition Ti+HA (22%).

**Table 1.** Electrochemical parameters obtained in 1.5 SBF at 36.5 and pH 7.4.

| Samples                     | Ecorr (V) | Icorr (A/cm²) | Ipass (A/cm²) |
|-----------------------------|-----------|---------------|---------------|
| C.P. Ti                     | -0.4956   | 1.801x10⁻⁵    | 1.594x10⁻⁵    |
| Ti + HA + AgNO₃             | -0.2944   | 4.700x10⁻⁵    | 1.120x10⁻³    |
| Ti + HA + AgNO₃ + PCL       | -0.1239   | 1.090x10⁻⁵    | 7.110x10⁻⁵    |

Ecorr: corrosion potential; Icorr: corrosion current density; Ipass: passivation current density.
Xue and collaborators (2004) used a plasma spraying coating technique for coating the titanium with hydroxyapatite and, in addition, incorporated Ag⁺. By the diffusion halo trials and XRD they observed an excellent inhibiting capability in silver against gram-positive bacteria⁴⁰, ⁴¹.

The inhibitory capacity of silver, observed by the higher efficiency of the conditions doped with AgNO₃, was characterized by this metal having a positive charge, which in contact with cell membranes that have negative charges, adhere to their surface, causing cell death and the total or partial elimination of bacteria⁴¹, ⁴². For the samples Ti+HA+AgNO₃+PCL, a 42% decrease in the amount of live bacteria in the supernatant liquid was observed.

Without the polymer coating (Ti+HA+AgNO₃) the bacterial viability was reduced by 81%, suggesting that the coating with PCL promotes a slower release of AgNO₃. As expected, the presence of silver nitrate in Ti+HA+AgNO₃ and Ti+HA+AgNO₃+PCL significantly reduced the bacterial viability in the medium due to the release of silver ions. However, the PCL-coated sample showed a lower efficiency in 24 hours. The polarization curves for the condition Ti+HA+AgNO₃+PCL presented a behavior which suggests that PCL promotes a release of silver ions in the culture medium, which can be considered favorable in cases of prolonged treatment.

Figure 7 presents the biofilm formation in the substrates, using the PE plate as a positive control for bacterial biofilm adhesion. In this case, PCL coating contributed to the reduction of biofilm formation compared to the sample Ti+HA+AgNO₃. This can be attributed to the alteration of the topography of the surface after polymer coating, since the formation of biofilm is favored by roughness, which increases the retention of bacteria on the surface.

The first stage of biofilm formation corresponds to the interaction and adhesion between the cells and the substrate, with roughness and surface free energy being identified as the factors that exert the greatest influence in this process⁴¹, ⁴⁴. In the micrographs presented in Figures 1 and 2 it is possible to observe that titanium disk coated with PCL presents a smoother surface compared to the sample Ti+HA+AgNO₃, making it difficult to retain bacteria.

The effect of surface hydrophilicity on the reduction of biofilm adhesion/formation can be observed by comparing the samples C.P. Ti and Ti+HA. After coating C.P. Ti with HA, phosphate and hydroxyl groups were introduced, which are highly hydrophilic, facilitating the formation of a water film on the surface of the substrate and consequently hindering the direct interaction between it and the bacterium.

In the present study, the antimicrobial effect of silver on the reduction of biofilm formation can be observed by comparing samples Ti+HA and Ti+HA+AgNO₃. Thus, the reduction of bacterial viability and biofilm formation in relation to the control indicates that the samples studied present a potential application for implants, due to the lower probability of rejection caused by the incidence of infection.

4. Conclusions

The microstructural characterization of Ti+HA+AgNO₃ and Ti+HA+AgNO₃+PCL proves the formation of a coating on the surface of the metallic substrate. The results from the XRD analysis confirm the typical peaks of α phase (compact hexagonal structure), hydroxyapatite, silver and PCL. The polarization curves reveal an improvement in the resistance to corrosion after doping with silver nitrate, and Ti+HA+AgNO₃+PCL exhibited better resistance to corrosion. An 81% reduction in the amount of live bacteria in the supernatant liquid was observed in the sample Ti+HA+AgNO₃, and a 42% reduction was observed in the sample CP Ti+HA+AgNO₃+PCL. The bactericidal effect of the doping which was carried out is thus proved. Comparing the results obtained with and without coating, it is possible to note that the presence of AgNO₃ was effective in reducing the amount of live bacteria present in the supernatant, as well the amount of live bacteria which adhered to the material’s surface. Furthermore, the polymer coating was capable of releasing the bactericidal agent, as proved by the corrosion assay and bacterial culture assays, suggesting the extension of the effect of the bactericidal agent. Thus, the Ti+HA+AgNO₃+PCL samples presented satisfactory results, such as corrosion resistance in bodily fluids, bone similarity (due to presence of HA), biocompatibility and bactericidal action, desirable characteristics for the application in medical implants.

5. Acknowledgements

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