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Dedicated to Professor Ionel Haiduc
on the occasion of his 80th anniversary

BIOMIMETIC HYDROXYAPATITE-SILVER COATINGS ON TITANIUM SURFACES

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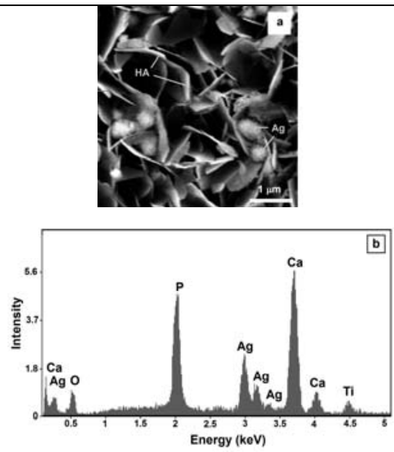
This study uses an *in vitro* experimental approach to investigate the possibility of obtaining hydroxyapatite-silver thin layers on pure titanium implants using a modified coating method. This method is based on a combined strategy involving hydroxyapatite biomimetic deposition on titanium surface using a supersaturated calcification solution (SCS) combined with silver ions reduction and *in situ* crystallization processes on hydroxyapatite-titanium surface by sample immersing in $AgNO_3$ solution. The hydroxyapatite-silver deposits on titanium surfaces were investigated by scanning electron microscopy (SEM) coupled with X-ray analysis (EDX) and X-ray diffraction (XRD). The results obtained revealed that hydroxyapatite coatings on titanium surface were formed and the silver incorporated into the apatite layer. The hydroxyapatite-silver coated titanium implants were tested against *Staphylococcus aureus* and *Escherichia coli* bacteria and the obtained data were indicative of good antibacterial properties of the materials.



INTRODUCTION

Commercially pure titanium and titanium alloys are materials most usually used as implants in dentistry and orthopedics surgery due to their excellent mechanical behavior, superior corrosion resistance and significant biocompatibility.[1,2] The clinical achievement of titanium implants is related to their early osseointegration and bone regeneration, and it depends on their surface properties. It is known that titanium and titanium alloys are bioinert materials according to their surface oxides and the osseointegration of titanium occurs without a negative tissue response.[3] For this reason, many researches have been dedicated to finding alternative methods to accelerate and optimize osseointegration of implants based on titanium materials in order to ensure their mechanical, biological and morphological compatibility.[4]

In the last times, titanium implants have been coated with calcium phosphate layers mainly composed of hydroxyapatite. The hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$) is chemically similar to the mineral component of bones and teeth in mammals and it is used as synthetic biomaterial.[5,6] As a bioactive material, hydroxyapatite allows bone

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ingrowth and osseointegration when used in orthopedic and dental applications. Different methods have been developed for coating the titanium implants with hydroxyapatite: plasma spraying, sputter-deposition, sol-gel coating, electrophoretic deposition or biomimetic precipitation.[7-9]

An essential problem in using implants is the occurrence of bacterial infections when placed within the human body. This limitation can be overcome by modifying the implant surfaces by means of antibacterial coatings while maintaining the good biocompatibility. Silver, as its oxidation states (Ag^0, Ag^+, Ag^{2+}, Ag^{3+}), has much been recognized as having an inhibitory effect toward many bacterial strains and microorganisms present in medical processes.[10] The silver-based biomaterials have attracted much attention due to their perfect antibacterial activity and nontoxicity.[11,12] The silver incorporation into hydroxyapatite coatings is an alternative that can provide good antibacterial properties of these coatings. There are several methods to introduce silver into hydroxyapatite coatings, such as sol-gel technology, electrochemical deposition, plasma spraying, ion beam-assisted deposition, magnetron sputtering, microarc oxidation and laser deposition.[13-15]

Up to now there are no studies on biocomposites consisting of hydroxyapatite-silver coatings on pure titanium surfaces by biomimetic precipitation in supersaturated calcification solutions (SCS). Consequently, in the present paper a combined strategy involving biomimetic approach and silver reduction process has been advanced to deposit a hydroxyapatite-silver layer on titanium surface able to regenerate the natural bone and mitigate implant infection. The bactericidal activity of the hydroxyapatite-silver coated titanium implants against *Escherichia coli* (Gram-negative) and *Staphylococcus aureus* (Gram-positive) bacteria was investigated and the bactericidal ratios evaluated as the antimicrobial efficiency.

EXPERIMENTAL

Materials

$CaCl_2{\cdot}2H_2O$, $NaH_2PO_4{\cdot}H_2O$, $NaHCO_3$, $AgNO_3$, NaOH, acetone and ethanol were purchased from Sigma-Aldrich (Germany). All chemicals were reagent grade and used without any other purification. Experiments were performed in triply distilled water. Plate-shaped implants fabricated from commercially pure Ti (c.p. Ti) were used.

Procedure

Titanium plate-shaped samples were cleaned with acetone, ethanol and deionized water. Samples were then treated in 0.6 M NaOH solution at 160°C in a pressure chamber for 72 h, at heating rates of 5°C /min. After alkaline pre-treatment, samples were washed in deionized water for 5 min and finally heat-treated at 60°C for 4 h.

The supersaturated calcification solution (SCS) was prepared by dissolving $CaCl_2{\cdot}2H_2O$, $NaH_2PO_4{\cdot}H_2O$ and $NaHCO_3$ in deionized water. The ion concentrations of SCS solution are: 4.0 $Mmol{\cdot}L^{-1}$ Na^+, 5.0 $Mmol{\cdot}L^{-1}$ Ca^{2+}, 10.0 $Mmol{\cdot}L^{-1}$ Cl^-, 2.5 $Mmol{\cdot}L^{-1}$ $(H_2PO_4)^-$ and 1.5 $Mmol{\cdot}L^{-1}$ $(HCO_3)^-$, as presented elsewhere.[16,17]

The biomimetic method applied to coating titanium surface with hydroxyapatite layer consisted in immersion of metallic samples into SCS solution at 37 °C, for certain period of time. Then, in order to obtain a silver coating, the titanium samples coated with hydroxyapatite layer were rapidly immersed into 50 mL of freshly prepared 0.5 M $AgNO_3$ solution of pH = 6.5 at 22°C, for a given period of time. Finally, the samples were taken off and carefully rinsed with deionized water followed by drying in air at 60°C for 24 h.

Characterization of samples

The morphology and chemical composition of samples were studied by scanning electron microscopy (SEM) coupled with energy dispersive X-ray spectroscopy (EDX) with QUANTA 200 3D microscope (FEI, Netherlands). Gold sputtering was used to make the coating surfaces conductive for the SEM investigations. The coating formed on titanium support was characterized by X-ray diffraction (XRD) with X'PERT PRO MRD diffractometer (PANalytical, Netherlands), using monochromatic CuKα radiation (λ = 0.15418 nm).

Evaluation of antibacterial activity

The spread plate method was used to analyse the antibacterial activity of hydroxyapatite-silver coatings. The tests were performed according to the biological standard methodology.[18] Two types of bacteria, *Escherichia coli* (Gram-negative) (ATCC 25922) and *Staphylococcus aureus* (Gram-positive) (ATCC 6538), were used in antibacterial experiments. The bacteria were counted through colony-forming units (CFUs) and bactericidal ratio (BR%) was calculated according to the following equation:

$$BR\% = \frac{CFUs_{(1)} - CFUs_{(2)}}{CFUs_{(1)}} \cdot 100 \tag{1}$$

where $CFUs_{(1)}$ is number of colonies in the control group (CFUs/ml), and $CFUs_{(2)}$ is number of colonies in the analyzed sample (CFUs/ml).

RESULTS AND DISCUSSION

In the present study, the biomimetic method was applied to deposit the hydroxyapatite and silver on the titanium surface. The biomimetic treatment of pure titanium implants consists of three steps: alkaline/heat treatment in NaOH solution, biomimetic treatment in supersaturated calcification solution (SCS) and immersion into $AgNO_3$ solution.

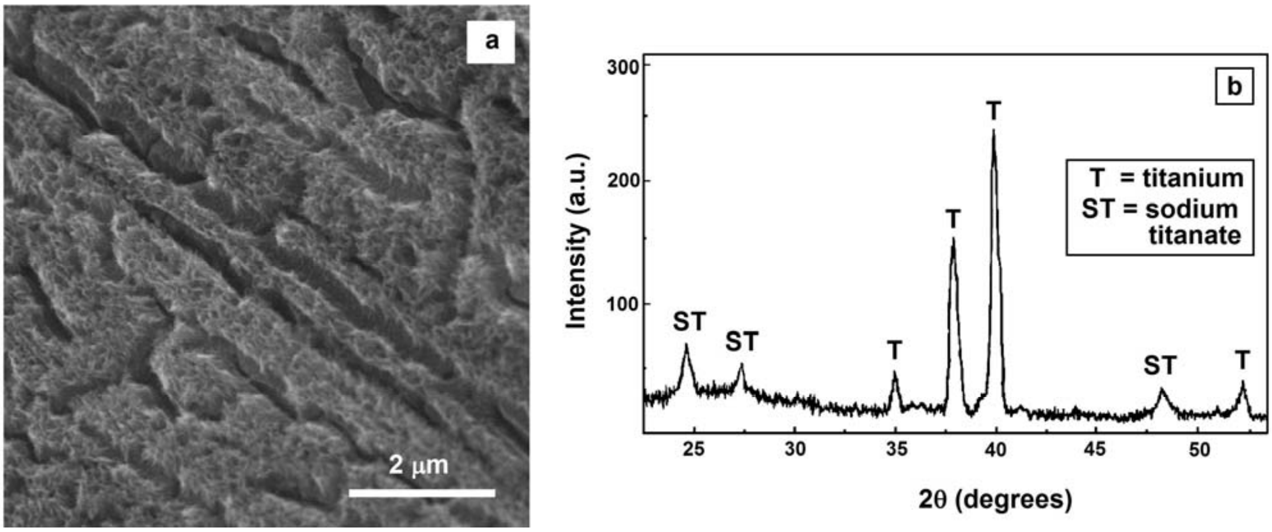

Fig. 1 – SEM micrograph (a) and XRD pattern (b) of the titanium surface after alkaline/heat treatment.

The hydroxyapatite and then hydroxyapatite-silver layers formed on titanium surfaces after biomimetic treatment in SCS and $AgNO_3$ solutions were examined by SEM-EDX and XRD methods.

After alkaline/heat treatment, the crystalline sodium titanate $Na_2Ti_5O_{11}$ was formed on the titanium surface, as presented in our previous studies.[9,19] The formation of the sodium titanate was confirmed by SEM and XRD investigations. SEM image of the titanate layer on titanium surface depicted in Fig. 1a indicates the most of the crystals to show the wire-like morphology. The XRD pattern (Fig. 1b) of the sample exhibits planes corresponding to the sodium titanate and titanium phases. The diffraction peaks at 2θ = 24.5º, 27.4º and 48.1º could be identified, in good agreement with a titanate structure (JCPDS card no. 11-0289).

In the SCS solution, where the degree of supersaturation of calcium and phosphate ions is high, some processes are developed. Thus, the sodium titanate releases Na^+ ions exchanged with the H_3O^+ ions in the solution to form Ti–OH groups on titanium surface. These Ti–OH groups are combining with Ca^{2+} ions in the SCS solution to form amorphous calcium titanate. Later, this calcium titanate is supposed to combine with phosphate ions in the solution to form intermediate precursor polymorphs such as amorphous calcium phosphate, octacalcium phosphate, or dicalcium phosphate dihydrate, with a low Ca/P ratio, as mentioned in literature.[5] The calcium phosphate transforms then into hydroxyapatite, which exhibits a Ca/P ratio of around value of 1.67.[20] The main reaction involved in the formation of hydroxyapatite during the biomimetic process can be expressed as follows:

$$10Ca^{2+} + 6PO_4^{3-} + 2OH^- \rightarrow Ca_{10}(PO_4)_6(OH)_2 \downarrow \quad (2)$$

The SEM micrograph (Fig. 2a) of a titanium sample stored in SCS solution for 96 h shows that the titanium surface is well enough covered with a hydroxyapatite layer. It can be observed that the crystalline apatite layer contains numerous crystals of thin plate morphologies. The EDX spectrum (Fig. 2b) is seen to exhibit pronounced Ca and P signals characteristic of the hydroxyapatite. The estimated Ca/P molar ratio of the crystals was of 1.673, which corresponds to the stoichiometric hydroxyapatite.[20]

The formation of the hydroxyapatite on titanium surface was confirmed by the X-ray diffraction analysis. The XRD pattern in Fig. 3a indicates that the crystals deposited onto titanium surface after soaking in SCS solution have the most intense peaks at 2θ values of about 25.6°, 31.5°, 32.7°, 34.3°, 39.6° and 46.7° representing the reflexion planes (002), (211), (300), (202), (310) and (222), which are similar to the standard hydroxyapatite with hexagonal crystal system and $6_{3/m}$ space group (JCPDS Data Card 09-0432).

After hydroxyapatite coating formation, the samples were immersed into $AgNO_3$ solution for a given time. During this stage, silver ions are transferred from the solution to the titanium surface (covered with a hydroxyapatite layer) where their reduction takes place. In the $AgNO_3$ solution containing silver ions, the silver crystals occur by reducing Ag^+ ions by the electron transfer ($Ag^+ + 1e^- \rightarrow Ag^0$) on the surface of hydroxyapatite. The hydroxyl groups on the surface of hydroxyapatite act in this process both as a reducing and a binding agent, as mentioned in literature.[21]

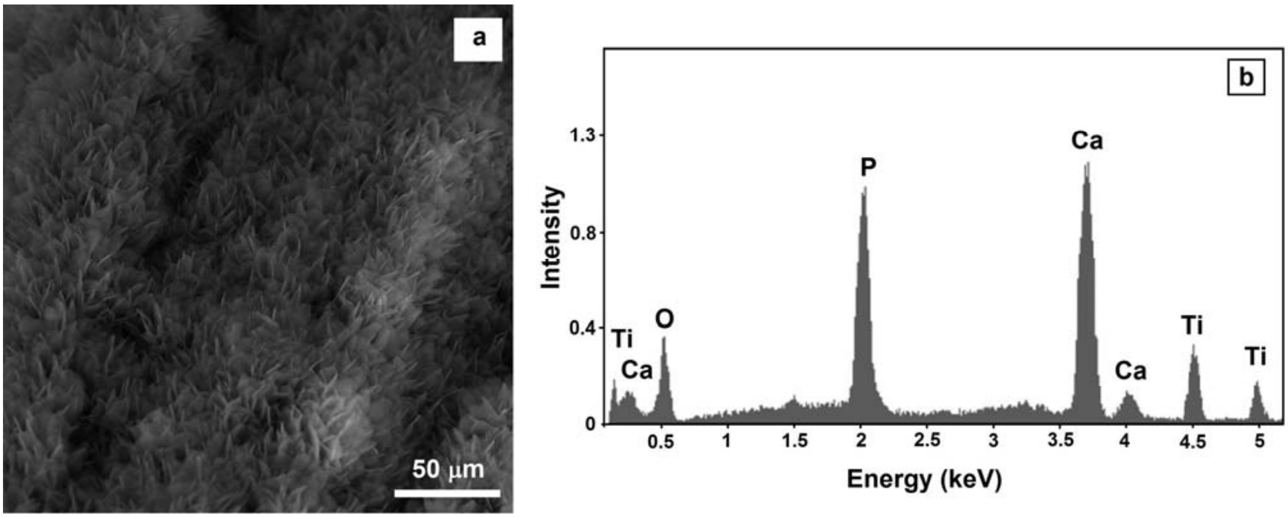

Fig. 2 – SEM image (a) and EDX spectrum (b) of the hydroxyapatite layer deposited on titanium surface after 96 h soaking into SCS solution at 37 °C.

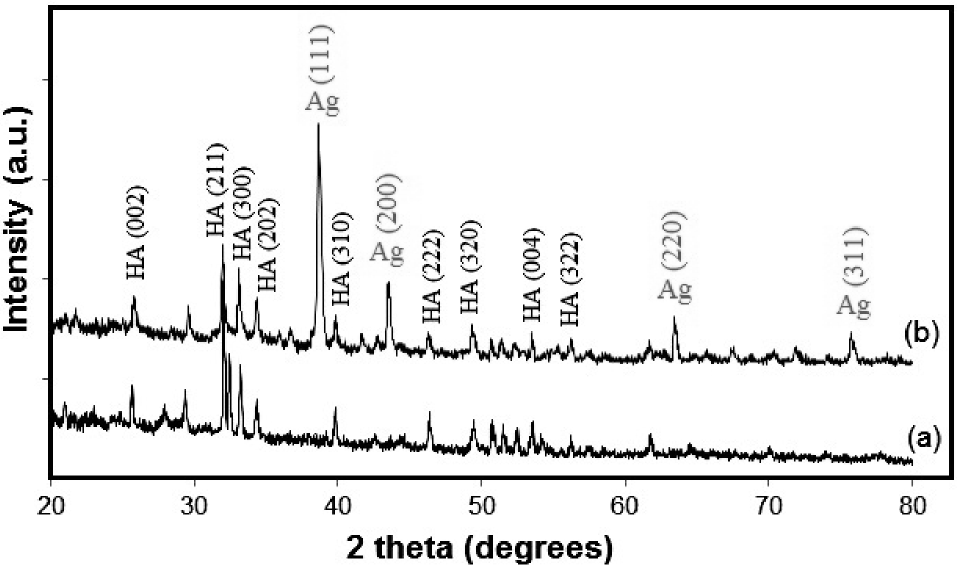

Fig. 3 – XRD patterns of the hydroxyapatite/titanium (a) and hydroxyapatite/silver/titanium (b) samples.

The XRD analysis of hydroxyapatite-silver coatings confirms the presence of silver in hydroxyapatite crystalline coatings (Fig. 3b). The diffraction peaks are noticed at about 38.3°, 44.6°, 64.4°, and 77.7° representing the (111), (200), (220) and (311) Bragg's reflections in the face centered cubic (fcc) phase structure of silver nanoparticles (JCPDS card no. 04.0784). As shown in SEM micrograph (Fig. 4a) of a titanium sample stored in $AgNO_3$ solution for 24 h, the spheroidal aggregates containing silver nanoparticles were formed on the titanium surface among hydroxyapatite (HA) crystals. The EDX results are also indicative of the silver particles formation. Figure 4b shows the EDX spectrum of hydroxyapatite-silver layer deposited on titanium surface, confirming that the coating contains silver (existing in nanoparticles and spherical aggregates), and Ca, P and O (in apatite crystals) atoms. The peaks at 2.9, 3.2 and 3.3 keV correspond to the binding energies of Ag Lα, Ag Lβ and Ag $L\beta_2$ respectively, as mentioned in literature.[22] The Ca/P molar ratio of apatite crystals was of 1.678, which corresponds to the stoichiometric hydroxyapatite.[20]

Figure 5 shows the schematic diagram illustrating the hydroxyapatite mineralization and silver particles formation on the titanium surface during immersion in SCS and $AgNO_3$ solutions.

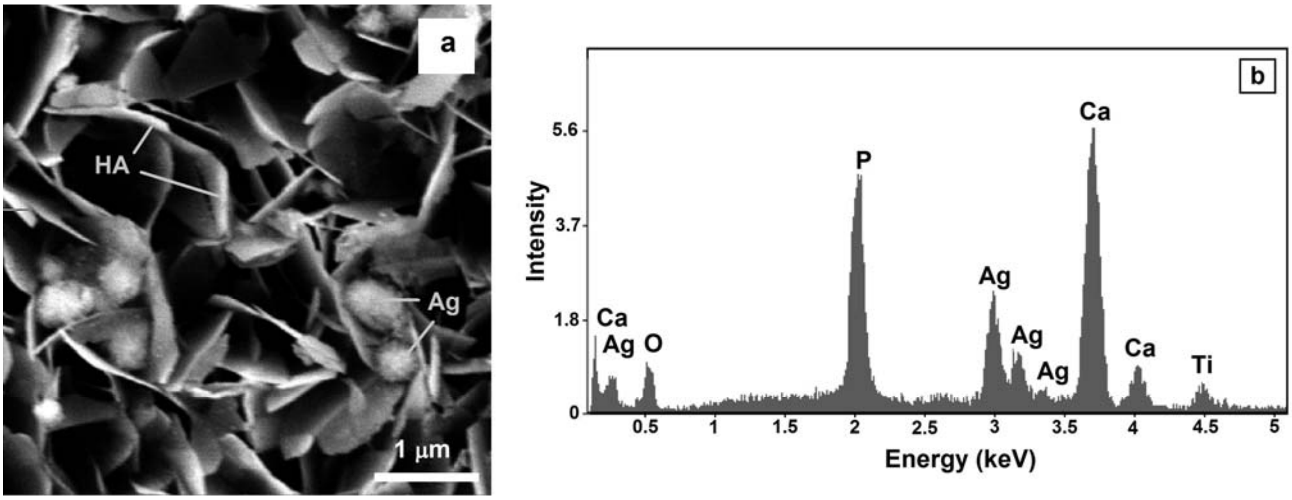

Fig. 4 – SEM image (a) and EDX spectrum (b) of the hydroxyapatite-silver layer deposited on titanium surface after 96 h soaking into SCS solution at 37 °C and after 24 h soaking into $AgNO_3$ solution at 22 °C.

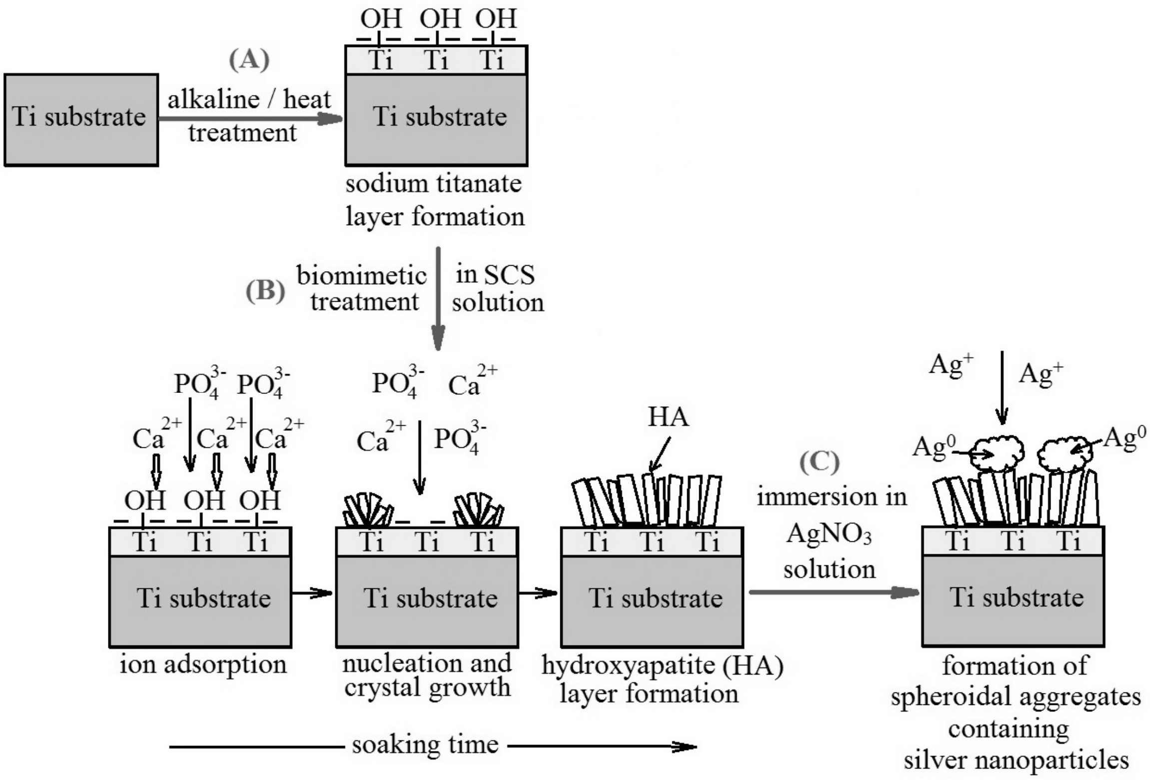

Fig. 5 – Schematic representation of the hydroxyapatite coating and silver particles formation on titanium surfaces after immersion into SCS and $AgNO_3$ solutions.

The bactericidal activity of the hydroxyapatite-silver coatings on titanium implants was evaluated by a spread plate method. Two kinds of bacteria, *Escherichia coli* (Gram-negative) and *Staphylococcus aureus* (Gram-positive), were used in antibacterial experiments. The obtained results indicate that about 86.3% *Staphylococcus aureus* and more than 98.1 % *Escherichia coli* were killed after 24 h of incubation. The inhibitory activity of the silver in the hydroxyapatite coatings on titanium surface is higher in case of Gram negative bacteria, as mentioned in literature.[23] These results would suggest that the antimicrobial effects of the silver might be associated with characteristics of certain bacterial species. The possible mechanism of action of silver as an antibacterial agent in hydroxyapatite-silver coatings could be due to the interaction of silver with the cell membrane of the organism, causing thus the structural damage and finally cell death. Gram-positive and gram-negative bacteria have different membrane structures, the most distinctive difference being the thickness of the peptidoglycan layer.[24] The lower efficacy of the silver against *Staphylococcus aureus* may be explained just by this difference in the cell membrane structures. Finally, the conclusion can be drawn that the hydroxyapatite-silver coated titanium implants exhibited a good antibacterial property.

CONCLUSIONS

In the present study the biomimetic method combined with reduction process and *in situ* crystallization was applied to co-deposit the hydroxyapatite crystals and silver nanoparticles on the pure titanium surface by means of metallic samples treatment in SCS and $AgNO_3$ solutions. The obtained results have shown that hydroxyapatite layers were produced on metallic surfaces with silver incorporated on these apatite layers. By the deposition of silver nanoparticles on the surface of hydroxyapatite coated titanium implants the antimicrobial activity against both *Escherichia coli* and *Staphylococcus aureus* bacteria has attained high values. The inhibitory activity of the silver in the hydroxyapatite coatings on titanium surface is higher in case of Gram negative bacteria. The titanium pieces covered with thin hydroxyapatite-silver layers could be applied as antimicrobial biomaterials for various purposes like orthopedic and dental implantation.

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