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## PhD Thesis Summary

**Studies and research on hydroxyapatite-based coatings development with high osseointegration properties and antibacterial ability using electrochemical techniques**

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## Abstract

This doctoral thesis addresses an important topic in biomaterials engineering, namely surface biofunctionalization of implantable medical devices in order to enhance the osseointegration capacity and reduce the risk of failure due to intrahospital infections.

In this direction, hydroxyapatite-based coatings doped with metal ions were developed in order to accelerate the osseointegration processes and induce antibacterial properties through electrochemical deposition techniques. Nonetheless, the electrochemical parameters involved in the electrochemical deposition techniques used (time, concentration and electrolyte's  $pH$ ), were optimized in order to obtain hydroxyapatite-based coatings undoped and doped with Sr and/or Ag with similar thicknesses and optimal concentration of the doping elements, in order to obtain superior characteristics and without cytotoxic effects.

These coatings were extensively characterized in terms of morphology, elemental and phasic composition, structural parameter values, crystallinity, crystallite size, chemical bonds, topography, wettability, adhesion, roughness and coating thickness. Subsequently, the hydroxyapatite-based coatings were tested *in vitro* in terms of electrochemical behavior and bioactivity in acellular biological media, but also the cellular response on preosteoblasts cell cultures (viability, proliferation, morphology and differentiation).

The obtained results showed that the features of the hydroxyapatite-based coatings undoped and doped with Sr and/or Ag obtained in electrolyte with  $pH$  5, can be modulated by controlling the electrochemical parameters involved in the deposition process, leading to coatings with superior properties that can be used in medical applications.

**Keywords:** hydroxyapatite, silver, strontium, electrochemical deposition, codoping, electrolyte  $pH$ , biocompatibility

## Introduction

Biomaterials have gained particular interest following the current clinical requirements determined by the ability to address medical conditions, but also due to the increment of the average age. The commercialization of implants is based on research and development of new materials by involving scientists from fields such as chemistry, biology, medicine and materials engineering.

The success of an implant depends on the process of bone remodeling and its capacity for osseointegration. These processes are dependent on the patient's age, medical conditions, but also on the prevalence of bacteria within the hospital units that lead to the occurrence of implant-related infections.

Nowadays we are witnessing to an increment of the patients over 50 years old that are suffering from hard bone tissue associated conditions. Osteoporosis is one of the bone tissue diseases that has a direct impact on the bone remodeling process and implicitly the patient's life, affecting approximately 22% of the population at European level.

Infections associated with implantable medical devices are also a problem that doctors face after surgery. Gram-positive (*S. aureus*) and Gram-negative (*E.coli*) bacteria can affect the implant starting the first days after implantation, with the risk of occurrence even after 2 years, due to the displacement of bacteria by the blood.

Thus, the development of implants that present both bioactive and antibacterial characteristics but also properties that can support the osteoblasts proliferation is a clinical requirement necessary to reduce surgical interventions, but mostly to improve the quality of life of patients.

The biomaterials market is currently dominated by metallic biomaterials, closely followed by ceramic ones. Among the metallic biomaterials, the titanium-based alloys are still the most used one, due to their superior properties compared to other classes of metallic biomaterials.

Surface modification or biofunctionalization of titanium-based implants is one of the main research directions within this field. Among the most used biomaterials for coating titanium-based metallic implants are calcium phosphates and especially hydroxyapatite. The presence of hydroxyapatite in the structure of hard bone tissues has led researchers to a well-defined goal, namely, to develop new hydroxyapatite-based coatings for metallic implants made of titanium-based alloys.

In addition to its superior properties, the structure of hydroxyapatite allows the substitution of calcium ions with other ions, thus improving the characteristics and osseointegration capacity of the implant.

The electrochemical methods allow the development of hydroxyapatite-based coatings doped with different types of elements that support the proliferation and osteogenic differentiation of cells, while also inducing an antibacterial effect. Among the electrochemical techniques, the pulse technique allows to attain coating with superior properties due to the electrochemical parameters involved in the deposition process that can be easily controlled.

## Part I – Literature study

### Chapter 1. Literature study regarding the applicability of biomaterials in medicine

#### 1.1. Types of biomaterials used in medicine

The field of biomaterials has been an active and continuously expanding field for about 70-80 years, significantly influencing human health, economics, and other scientific fields. Biomaterials and medical devices are currently used in areas of medicine such as cardiology, orthopedics, dentistry and reconstructive surgery. The most convincing argument of the biomaterials benefits within the society consists in the millions of lives that have been saved, while for several millions more the quality of life has been substantially improved.

The importance of biomaterials in the development of implantable medical devices depends on factors such as: a) obtaining, processing and developing new biomaterials with improved characteristics, b) a well-developed design technique, c) preclinical tests – *in vitro* tests on specific cell cultures, and *in vivo* tests on animal models (mice, rabbits, etc.); clinical trials - human trials, d) obtaining the necessary approvals and certifications in accordance with the regulations of the Food and Drug Administration (FDA) from USA or from the European Union (EU-MDR), e) involving the industry to enable the products development and commercialization [3].

### Chapter 2. Biofunctionalization of metallic biomaterials with calcium phosphate-based osteoconductive coatings

#### 2.1. The necessity of using osteoconductive coatings in medical applications

A current trend to increase the biocompatibility of implants and to meet as many clinical requirements as possible consists in deposition of hydroxyapatite (HAp) on metallic substrates. This concept has been introduced mainly to achieve a better integration of the implants. Thus, the most important arguments underlying the application and use of coatings in medical applications are found as follows:

- the substrate contains elements that, through their release, can induce cytotoxicity, adverse or allergic reactions;
- the coating protects the base material from the environment in which it was designed to function and increases its chemical and mechanical stability and service life;
- the coating improves the mechanical integrity, indicating that the coated products are more resistant (for example, to wear);
- modification of the substrate/base material surface properties, such as biocompatibility, translucency, electrical conductivity, etc.;
- changing the release profile of active substances, such as growth factors, elements with antibacterial/antifungal effect, etc.

#### 2.2. Classification of the deposition methods

The scientific literature presents a variety of technologies under different names and classifications, so the classification of hydroxyapatite-based coatings deposition techniques can be made according to several criterions. The most widely used classification of the main deposition techniques used to obtain hydroxyapatite-based coatings is made according to the phenomenon involved in the deposition process.

## Part II – Experimental research

### Chapter 3. Materials and characterization methods of hydroxyapatite-based coatings

#### 3.1. The objectives of the doctoral thesis

According to the literature data presented in Chapters 1 and 2, the electrochemical method for obtaining HAp-based coatings is a method that allows the deposition of coatings with superior properties by easily controlling the electrochemical parameters involved in the deposition process.

The main objective of this PhD thesis consisted in the development of hydroxyapatite coatings with high osseointegration and antibacterial properties, by using the electrochemical deposition techniques. This desideratum can be achieved by doping the hydroxyapatite with metal ions that accelerate osseointegration processes (Sr) and induce antibacterial properties (Ag). The coatings developed in this PhD thesis can be used to improve the osseointegration capacity of metallic implantable medical devices, especially those made of Ti and Ti alloys.

To achieve the main objective of the thesis, other specific objectives were considered:

- Development of hydroxyapatite coatings undoped and doped with Sr and/or Ag with similar thicknesses, and concentrations of the doping elements in optimal amounts in order to reach superior characteristics and at the same time not to induce cytotoxic effects;
- Optimizing the electrochemical deposition parameters in order to achieve the first specific objective;
- Complex and thorough characterization of the hydroxyapatite coatings undoped and doped with Sr and/or Ag in terms of morphology, elemental and phase composition, structural parameter values, crystallinity, crystallite size, chemical bonds, topography, wettability, roughness and coating thicknesses;
- *In vitro* testing regarding the electrochemical behavior and bioactivity in acellular biological media and of cellular response on preosteoblast cell cultures (viability, proliferation, morphology and differentiation).

#### 3.2. Materials

In this PhD thesis, an titanium alloy (Ti6Al4V) and pure titanium (cp-Ti, grade 2), both purchased from BibusMetals, AG (Germany) as bars with a diameter of 20 mm were used as substrates for the hydroxyapatite-based coatings. To obtain the experimental samples, discs with a thickness of 2 mm were cut from titanium and titanium alloy bars.

*Table 3.1. Chemical composition of pure titanium according to the manufacturer*

	Material	
	Ti6Al4V ELI	cp-Ti (grad 2)
Chemical composition (wt.%)	Al	5.77
	V	3.80
	Fe	0.11
	C	0.019
	N	0.002
	H	0.006
	O	0.109
	Residues	max. 0.4
Titanium	balance	
Manufactured in accordance with the following standards	ASTM F136-13 ISO 5832-3:96 ASTM 348-13	ASTM B348-10 ASTM F67

### 3.3. Characterization methods

The hydroxyapatite coatings undoped and doped with Sr and/or Ag were characterized in terms of morphology, elemental and phase composition, chemical bonding, roughness and layer thickness, adhesion and wettability, and surface energies. In the following, each characterization method is briefly described, as well as the technique used for the investigation of the experimental materials developed in the framework of this doctoral thesis.

### 3.4. Test methods

The hHAp-based coatings undoped and doped with Sr and/or Ag were tested in terms of electrochemical behavior, bioactivity through biomineralization tests in simulated body fluid (SBF) and biodegradation in phosphate buffer solution (PBS), being also highlighted the biological properties through extensive in vitro tests on cell cultures.

## Capitolul 4. Chapter 4. Development and characterization of the bioactive, osteoconductive and antibacterial hydroxyapatite-based coatings

### 4.1. Introduction

The general objective was to develop a new technique to improve the bioactivity of Ti6Al4V alloys using the pulsed electrochemically assisted deposition technique to obtain hydroxyapatite (HAp)-based coatings doped with silver (Ag), as possible candidates in medical applications, due to the high osseointegration capacity and antibacterial effect. The specific objectives aimed to obtain undoped and Ag-doped hydroxyapatite-based coatings of similar thicknesses [115].

### 4.2. Sample preparation and development of the coating

The deposition of calcium phosphate coatings through pulsed electrochemical deposition (ED) on Ti6Al4V substrate were carried out in a standard three electrochemical cell: the Ti6Al4V alloy - working electrode (WE), a Pt plate with an area of  $\sim 1 \text{ cm}^2$  - auxiliary electrode (AE), and a calomel electrode (SCE) - reference electrode (RE).

The electrochemical deposition was carried out with a potentiostat/galvanostat, using the potentiostatic pulsed technique by applying a potential difference between the WE and the AE over several cycles; 1 cycle consists of an activation potential ( $E_{\text{ON}}$ ) of  $-2 \text{ V}$ , applied for  $0.5 \text{ s}$  ( $t_{\text{ON}}$ ) and a relaxation period in which the applied potential ( $E_{\text{OFF}}$ ) was  $0 \text{ V}$ , for  $1 \text{ s}$  ( $t_{\text{OFF}}$ ) [115]. Table 4.1 shows the sample codification and the chemical composition of the electrolytes used for calcium phosphate coatings that have a Ca/P molar ratio of 1.67, which is specific to stoichiometric hydroxyapatite. Briefly, the chemicals shown in Table 4.1 were subsequently dissolved in ultrapure water, and the final pH of the solution was adjusted to 4 by adding a very small volume (approx.  $2 \text{ mL}$ ) of nitric acid solution,  $1 \text{ M HNO}_3$ . The coatings were deposited at a temperature of  $75 \text{ }^\circ\text{C}$ .

*Table 4.1. Sample codification and chemical composition of the electrolyte used in electrochemical deposition [115]*

Coating description	Codification	Chemical composition (mM)			Ca/P ratio
		$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	$\text{NH}_4\text{H}_2\text{PO}_4$	$\text{Ag}(\text{NO}_3)$	
Ti6Al4V coated with hydroxyapatite	HAp	5	3	-	1.67
Ti6Al4V coated with hydroxyapatite doped with Ag	HAp-Ag	4.85		0.15	

Several samples of hydroxyapatite doped with Ag were made, aiming to obtain a similar thickness to that of the undoped samples, of approximately  $7 \text{ }\mu\text{m}$ . Therefore, based on the experiments carried out and considering the coating thickness, two deposition times were selected, namely  $90 \text{ min.}$  for the undoped HAp-based coatings and  $10 \text{ min.}$  for the ones doped with Ag, since the obtained coatings thicknesses are close, registering values of  $6.7 \text{ }\mu\text{m}$  for the

undoped HAp based coatings and 7.1  $\mu\text{m}$  for the ones doped with Ag [115].

### 4.3. Characterization of osteoconductive coatings

#### 4.3.1. Morphology

From a microscopic point of view, the SEM images revealed a plate-like morphology, characteristic of stoichiometric HAp (Ca/P ratio = 1.67), for both types of coatings.

It can also be observed that the preferential growth direction is perpendicular on the working electrode surface, this being the preferential direction in the crystallization [31,165,174,180].

Nonetheless, regarding the hydroxyapatite coatings doped with Ag, the morphology of the coatings consists in thinner and wider plate-like crystals, on which small bright gray particles are fixed, that can be attributed to Ag

#### 4.3.2. Elemental composition

The EDS spectra have confirmed the presence of calcium phosphates characteristic elements (Ca, P, O), of the Ag doping element, as well as those of the substrate (Ti, Al and V).

Table 4.3 shows the elemental composition of hydroxyapatite coatings and the Ca/P ratio.

Considering the mass concentration (wt.%) of Ag in the HAp-Ag coatings it can be stated that it exceeds the limit of 6 wt.%, registering a value of 7.3 wt.%.

It is observed that both coatings present a smaller Ca/P ratio than that of stoichiometric HAp (1.67), of 1.58 for HAp and 1.62 for Ag-doped HAp, respectively. It can also be observed that addition of Ag into the HAp has led to an increment of the Ca/P ratio.

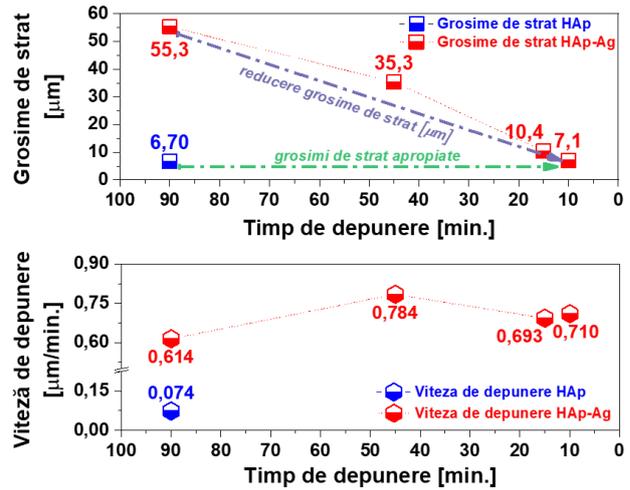


Figure 4.1. Variation of coating thickness depending on deposition time

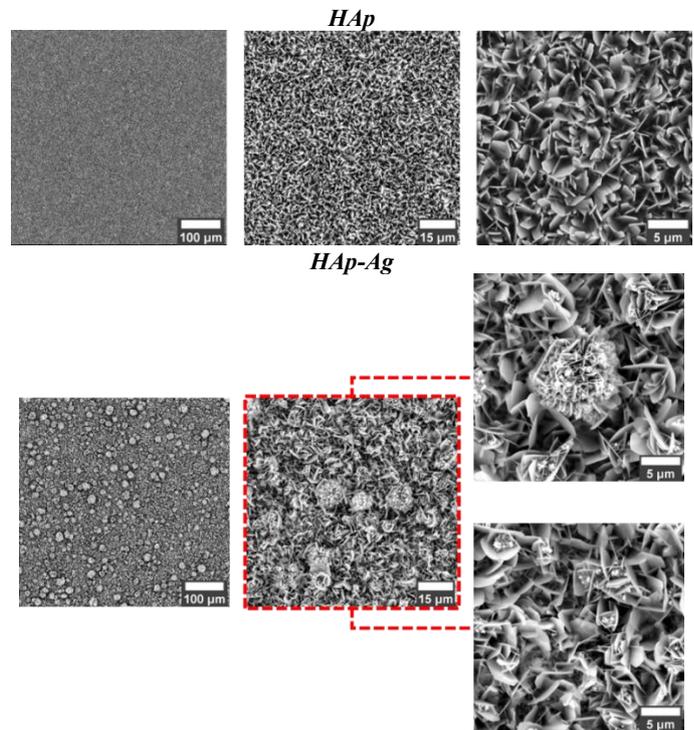


Figure 4.2. Morphology of undoped (HAp) and doped (HAp-Ag) hydroxyapatite coatings

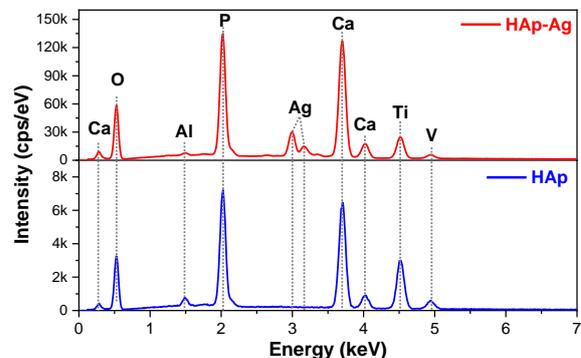
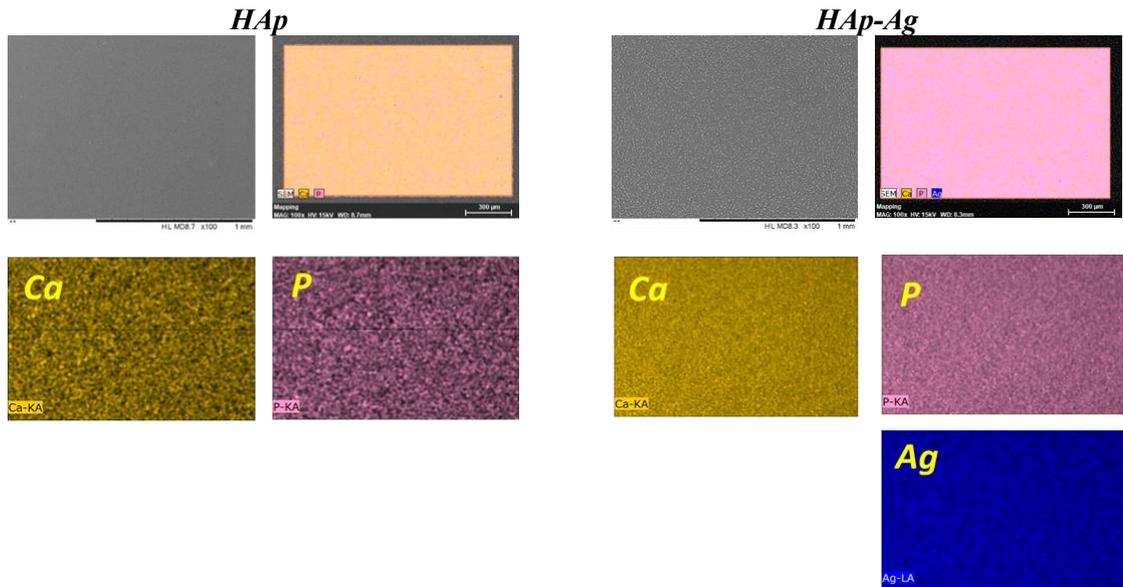


Figure 4.3. EDS spectra obtained for HAp and HAp-Ag coatings on Ti6Al4V substrate

**Table 4.3. Elemental composition and (Ca+Ag)/P ratio for the HAp-based coatings**

Elemental composition (at.% and wt.%)		HAp	HAp-Ag
Ca	at.%	61.2	59.3
	wt.%	67.1	61.9
P	at.%	38.8	38.1
	wt.%	32.9	30.8
Ag	at.%	0.0	2.6
	wt.%	0.0	7.3
<b>(Ca+Ag)/P ratio</b>		<b>1.58</b>	<b>1.62</b>

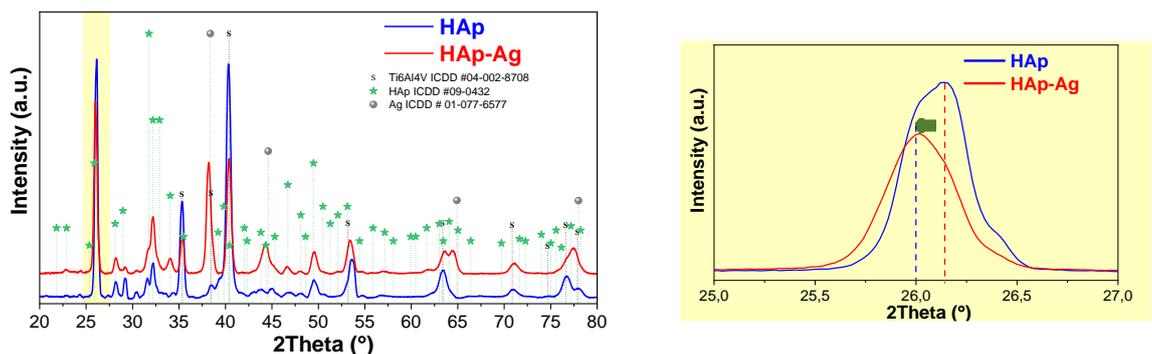
According to Figure 4.5, it can be seen that the characteristic elements (Ca and P) of the undoped HAp but also the doping element (Ag) are uniformly distributed throughout the investigated surface of the coatings.



**Figure 4.5. Distribution of constitutive elements (Ca, P and Ag) in the HAp and HAp-Ag coatings**

### 4.3.3. Phase composition

From the XRD diffractograms, it can be noted that both types of developed coatings are crystalline (Figure 4.6). The diffraction peak identified for both types of coatings are specific to hydroxyapatite according to the ICDD standard number 09-0432, thus confirming the presence of hydroxyapatite on the Ti6Al4V substrate by the potentiostatic pulsed technique. The addition of Ag in the HAp structure led to a decrement in the relative intensity of the diffraction peak associated to the (002) plane at  $2\theta$  of approximately  $26^\circ$ , suggesting a decrease of the hydroxyapatite crystallinity [218]. It has also been observed that after the addition of Ag in the HAp structure, the diffraction peak at  $2\theta$  of about  $26^\circ$  shifted slightly to the left, towards smaller  $2\theta$  angle values [219], denoting that the crystal lattice underwent changes of the lattice parameters.



**Figure 4.6. X-ray diffractogram of the HAp and HAp-Ag coatings**

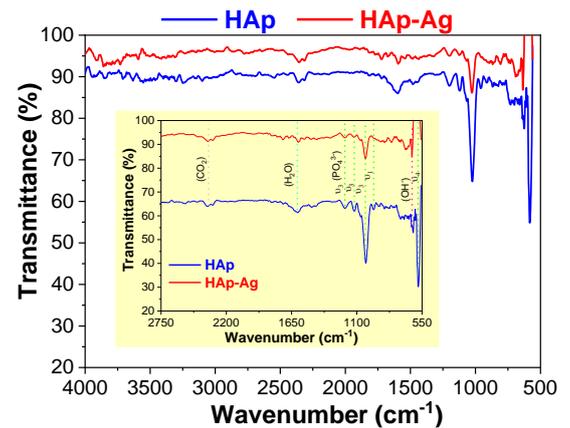
As can be seen from Table 4.4, from the point of view of lattice parameters it is observed that the coatings show similar values for the  $c$  axis, while for the  $a$  and  $b$  axis the values increased with the addition of Ag. At the same time, the crystallite size and crystallinity decreased after the addition of Ag in the HAp structure.

**Table 4.1.** Main structural parameters: lattice parameters, crystallite size and crystallinity

Sample	2 $\theta$ ( $^{\circ}$ )	Lattice parameters		Crystallite size (nm)	Crystallinity, $\chi_c$ (%)
		$a=b$ (nm)	$c$ (nm)		
HAp	26.10	0.951	0.685	35.88	29.07
HAp-Ag	26.02	0.962	0.685	21.23	22.50

#### 4.3.4. Chemical bonds

The FTIR spectra obtained for the hydroxyapatite-based coatings undoped and doped revealed the specific vibrational bands of the phosphate group ( $\text{PO}_4^{3-}$ ) in the wavenumber range 900 and 1200  $\text{cm}^{-1}$  [224]. The presence of  $\text{PO}_4^{3-}$  groups indicates the formation of an apatite-type structure. The peak at 630  $\text{cm}^{-1}$  is characteristic to the hydroxyl group ( $\text{OH}^-$ ) and confirms the presence of HAp. It can also be observed that after the addition of Ag, no shifts towards lower or higher wavenumbers of the peaks were identified.



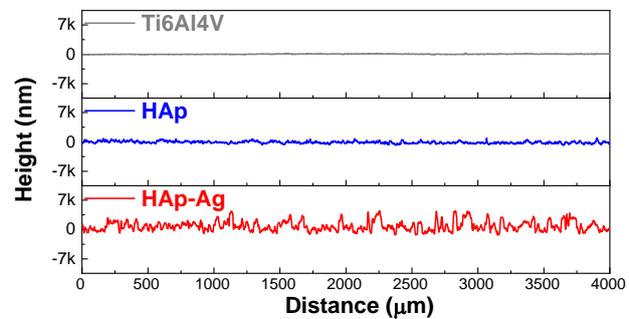
**Figure 4.7.** FTIR spectra of the HAp and HAp-Ag coatings

#### 4.3.5. Roughness and topography

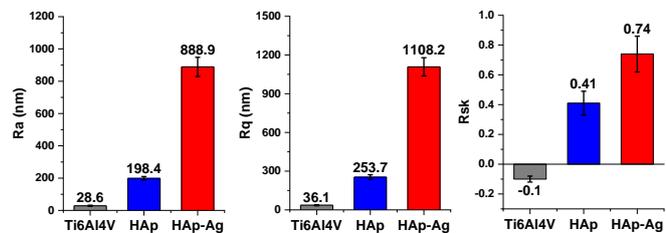
According to the obtained results, it can be observed that, in comparison with the Ti6Al4V substrate, the undoped and Ag-doped HAp coatings registered higher values.

Regarding the asymmetry, Rsk, it can be stated that the HAp and HAp-Ag coatings show positive values of this parameter, while the Ti6Al4V alloy substrate recorded a negative value of this parameter.

As can be seen the plate-like formations identified from the SEM analysis of the undoped HAp coatings show different sizes according to the AFM analysis (Figure 4.10). After the addition of Ag, the plate-like formations are larger with sharper edges compared to those observed for undoped HAp whose formations are much smaller.



**Figure 4.8.** Representative profiles for the investigated surfaces



**Figure 4.9.** Evolution of the main roughness parameters

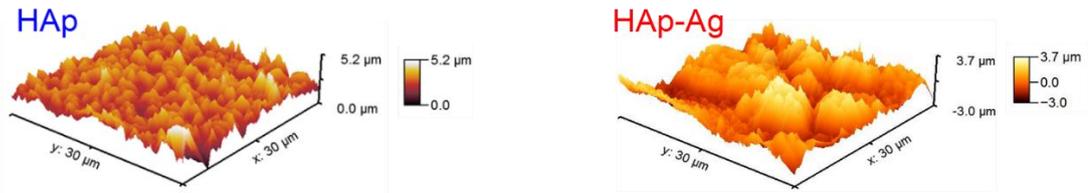


Figure 4.10. AFM images of the surface topography of the developed coatings

#### 4.4. Coating testing

##### 4.4.1. Bioactivity assays

According to the obtained results from the immersion tests in SBF, the Ti6Al4V alloy did not favor the precipitation of new calcium phosphates on the surface [115], thus presenting the weakest biomineralization capacity among the investigated materials (Figure 4.11).

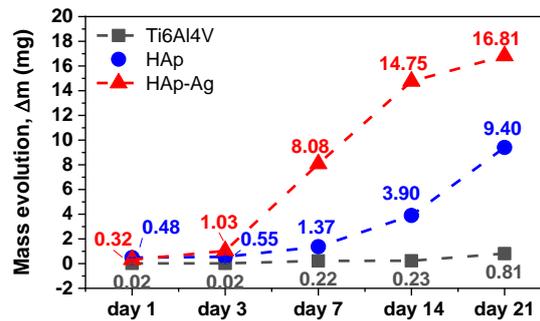


Figure 4.11. The evolution of the newly formed apatite mass on the surface of the experimental materials

The best biomineralization capacity was recorded for Ag-doped hydroxyapatite coatings, followed by the undoped hydroxyapatite coatings after 21 days of immersion in SBF [115]. Thus, based on the results obtained, it can be stated that the modification of the Ti6Al4V alloy surface with hydroxyapatite-based coatings undoped and doped Ag have led to the improvement of the biomineralization capacity, indicating that the addition of Ag into the HAp structure accelerates the precipitation of a new layer of apatite.

Also, regardless the coating type, Ag-doped or undoped HAp, a new apatite layer was formed on the surface exposed to SBF (Figure 4.12). After 21 days of exposure to the SBF testing environment, both coatings show newly formed hemispherical structures over the entire surface, which due to the accumulated internal stresses has generated cracks.

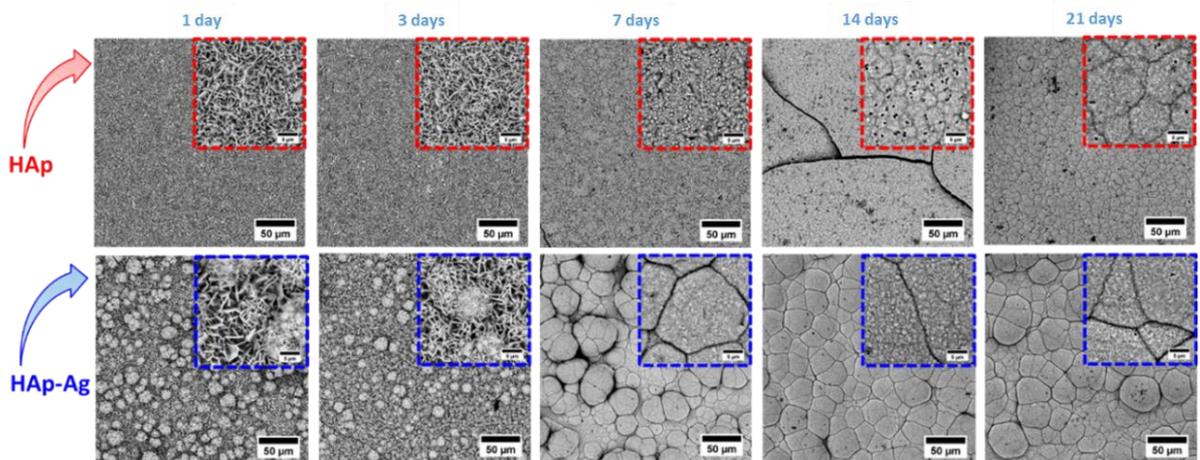


Figure 4.12. SEM images of undoped and Ag-doped HAp coatings after immersion tests in SBF

#### 4.4.2. Electrochemical behavior

Figure 4.14 shows the potentiodynamic curves of the investigated materials in the two testing media, while the main electrochemical parameters obtained from the Tafel extrapolation are shown in Table 4.6.

In the case of tests carried out in SBF, the most electropositive corrosion potential and thus the best electrochemical behavior is recorded in the case of Ag-doped hydroxyapatite (HAp-Ag), followed by HAp and Ti6Al4V. From the point of view of the corrosion current density, it is noted that the lowest value of this parameter and implicitly a better electrochemical behavior is presented by the HAp sample, followed by the uncoated alloy and by the HAp-Ag coatings which has a higher value of this parameter, most likely due to the metallic character of the doping element.

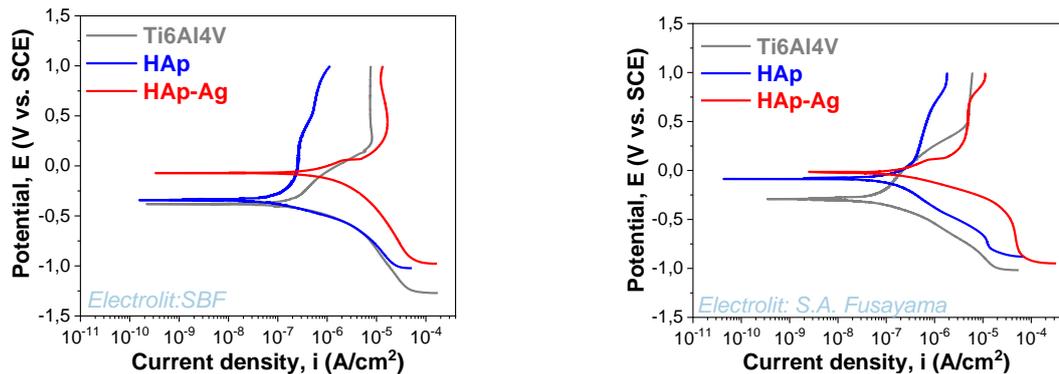


Figure 4.14. Potentiodynamic plots for the investigated samples in a) simulated body fluid - SBF and b) Fusayama artificial saliva

Table 4.6. Main electrochemical parameters of the investigated samples

Electrolyte	Sample	$E_{oc}$ (mV)	$E_{corr}$ (mV)	$i_{corr}$ (nA/cm <sup>2</sup> )
SBF	Ti6Al4V	-286.01	-283.37	83.75
	HAp	-7.13	-206.28	40.07
	HAp-Ag	-10.58	-56.64	780.80
SA Fusayama	Ti6Al4V	-22.15	-292.95	96.13
	HAp	115.32	-81.81	60.71
	HAp-Ag	74.07	-21.88	232.27

In the case of the tests carried out in Fusayama artificial saliva, an electrochemical behavior similar to that identified for the tests carried out in SBF is observed. Thus, the HAp-Ag coating presents the best electrochemical behavior identified by the most electropositive  $E_{cor}$ , followed by HAp and the uncoated Ti6Al4V alloy. The lowest corrosion current density was recorded in the case of the HAp sample which, from this point of view, has a better electrochemical behavior in artificial saliva compared to the other investigated samples.

#### 4.5. Conclusion

The potentiostatic pulsed electrochemical deposition is a technique that presents major advantages such as the deposition of doped coatings from a single electrolytic solution with different ionic species, and by changing the deposition parameters, coatings with desired/tunable properties can be obtained. However, it cannot be overlooked that in the case of the hydroxyapatite-based coatings, the deposition time was 90 minutes, which is quite long compared to the time used for the Ag-containing coatings. Also, in the case of the Ag-doped hydroxyapatite coatings, the appearance of those particle agglomerations as well as the high Ag concentration within the obtained layers, can generate unwanted effects such as cytotoxicity, which in the last instance can even lead to implant failure.

All of this, combined with the optimization in terms of deposition time and energy efficiency, have led to the orientation towards another electrochemical technique that can

simultaneously offer the necessary properties of the coatings obtained by the potentiostatic pulse technique, as well as the characteristics above mentioned.

## Chapter 5. The influence of electrolyte's pH on the physicochemical properties of the HAp coatings undoped and doped with Sr and/or Ag

### 5.1 Development of HAp coatings undoped and doped with Sr and/or Ag

The HAp-based coatings were obtained on a pure titanium (cp-Ti) substrate through the electrochemical method, by using the galvanostatic pulsed deposition technique [117]. This coating deposition technique was chosen because the activation and relaxation times involved in the deposition process can lead to a higher adhesion of the layers than other techniques. Furthermore, through this technique, the thickness of the coatings can be easily controlled.

Table 5.2 shows the molar concentration of the chemical compounds used to obtain the HAp-based coatings. To prepare the electrolyte, the precursors were subsequently dissolved in ultra-pure water (ASTM 1). In all cases the electrolytes were prepared so that the Ca/P ratio to be 1.67 [159].

*Table 5.2. Molar concentration of salts used to obtain the HAp-based coatings [117]*

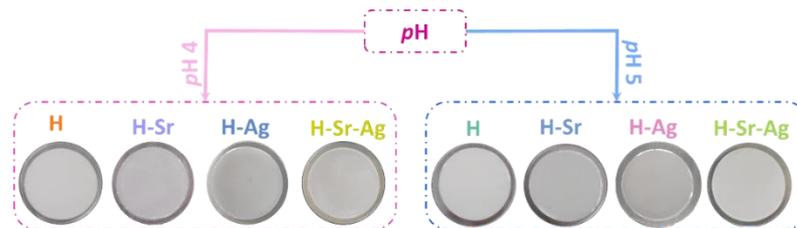
Samples codification	Chemical composition (mM)				(Ca+M)/P (M=Sr/Ag)	pH
	Ca(NO <sub>3</sub> ) <sub>2</sub> •4H <sub>2</sub> O	NH <sub>4</sub> H <sub>2</sub> PO <sub>4</sub>	Sr(NO <sub>3</sub> ) <sub>2</sub>	AgNO <sub>3</sub>		
H	10		-	-		4
H-Sr	9		1	-	1.67	
H-Ag	9.98	6	-	0.02		
H-Sr-Ag	8.98		1	0.02		5

The electrochemical parameters used to obtain the HAp-based coatings are presented in Table 5.3.

*Table 5.3. Electrochemical parameters used to obtain HAp-based coatings*

Sample codification	Deposition parameters					
	Cycle				Number of cycles; Deposition time	Temperature
	i <sub>ON</sub> (mA/cm <sup>2</sup> )	t <sub>ON</sub> (s)	i <sub>OFF</sub> (mA/cm <sup>2</sup> )	t <sub>OFF</sub> (s)		
H					900 cycles; 45 min	75 °C
H-Sr	-0.85	1	0	2		
H-Ag						
H-Sr-Ag						

In Figure 5.1. are presented the macroscopic images of the undoped hydroxyapatite (H) and doped with strontium (H-Sr), silver (H-Ag) and with strontium and silver (H-Sr-Ag). According to the macroscopic images, in all cases, and irrespective of the doping element or the electrolyte's pH, the coatings are uniform, without defects, and the entire surface exposed to the electrolyte was completely covered.

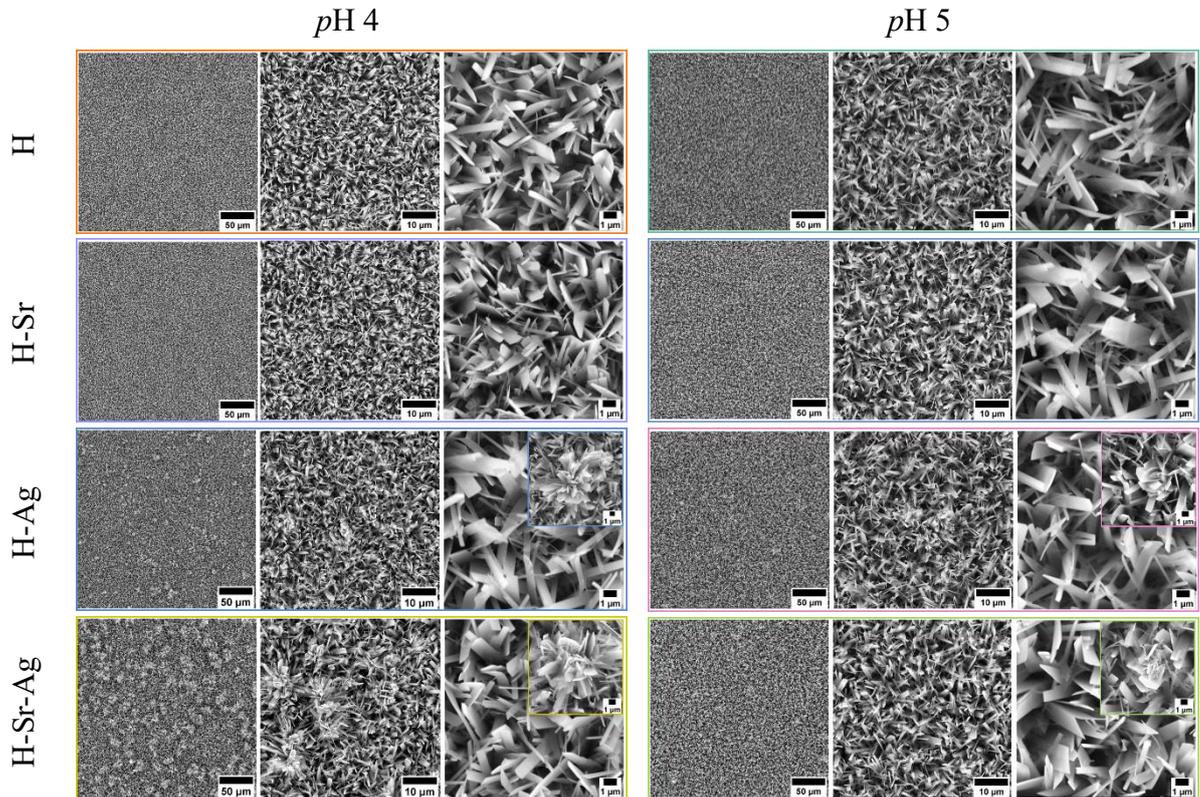


*Figure 5.1. Macroscopic images of the hydroxyapatite-based coatings undoped and doped with strontium and/or silver*

## 5.2 Preliminary characterization of the HAp-based coatings

### 5.2.1. Morphology of the HAp-based coatings

Figure 5.2. shows the morphology of HAp-based coatings undoped and doped with Sr and/or Ag obtained at  $pH$  4 and 5 at different magnifications. As can be seen in these images, the morphology of the coatings consists of ribbons-like crystals, very thin (nanometric level), wide and long regardless of the electrolyte's  $pH$  value in which they were obtained. At the same time, it can be observed that the coatings obtained in  $pH$  5 electrolyte present slightly narrower ribbons in contrast to those obtained at  $pH$  4.



**Figure 5.2.** Morphology of the HAp-based coatings undoped and doped with Sr and/or Ag

The substitution of calcium ions with strontium ions have led to narrower ribbons compared to those obtained in the case of undoped HAp, an aspect that can only be observed at higher magnifications. The substitution of  $Ca^{2+}$  ions with  $Ag^+$  ions have led not only to the formation of the ribbon-like morphology, but also to some flower-like agglomerations. Similar to the Ag-doped coatings are the co-doped ones, where the predominant morphology consists in ribbons and flower-like agglomerations. Nevertheless, the morphology of H-Ag and H-Sr-Ag coatings is strongly influenced by the  $pH$ , being observed that at  $pH$  5 the density of the flower-like agglomerations decreases significantly.

### 5.2.2. Elemental composition of HAp-based coatings

The elemental composition of the HAp-based coatings is shown in Figure 5.3 and Table 5.4. According to the EDS spectra the presence of all the elements involved in the deposition process can be observed regardless the electrolyte's  $pH$ : Ca, P and O for the undoped HAp-based coatings, Ag and Sr for the doped and co-doped HAp-based coatings, but also Ti originating from the cp-Ti substrate.

Considering the elemental composition shown in Table 5.4, the Ca/P ratios were calculated for the undoped HAp-based coatings and the  $(Ca+M)/P$  ratio for the HAp-based coatings doped and codoped with Sr and/or Ag, where M represents Sr and/or Ag [235,239] (Figure 5.4.). Thus, in all cases it can be observed that regardless of the electrolyte's  $pH$  the

coatings registered a ratio smaller than 1.67, suggesting that the obtained coatings are Ca deficient.

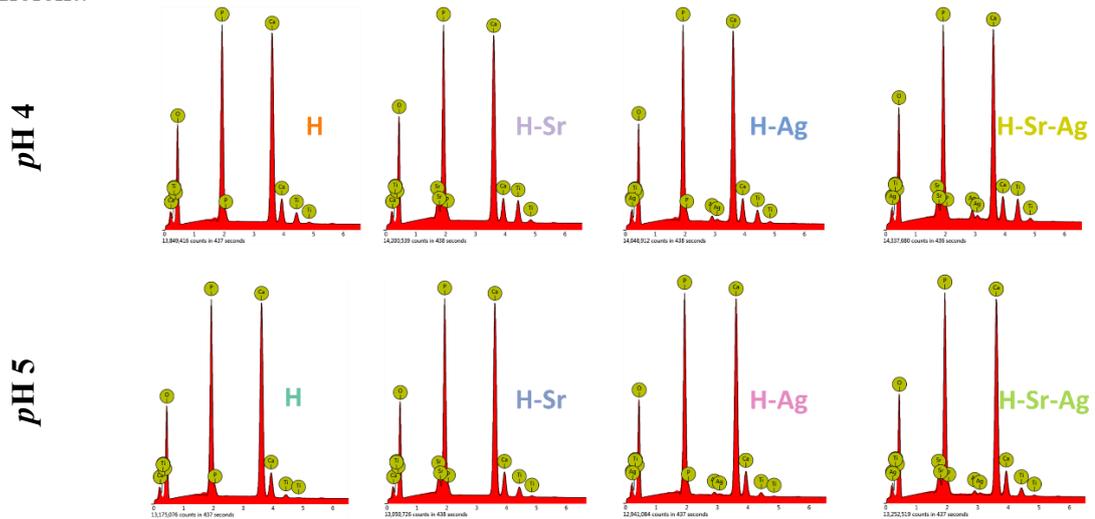


Figure 5.3. EDS spectra of the HAp-based coatings undoped and doped with Sr and/or Ag

Table 5.4 Elemental composition and (Ca+M)/P ratio for the HAp-based coatings [117]

pH	Sample	Chemical composition (at.%)				(Ca+M)/P (unde, M=Sr, Ag, Sr+Ag)
		Ca	P	Sr	Ag	
4	H	60.70	39.30	-	-	1.54
	H-Sr	55.62	40.85	3.52	-	1.45
	H-Ag	60.17	38.16	-	1.67	1.62
	H-Sr-Ag	54.88	39.89	3.43	1.80	1.51
5	H	61.18	38.82	-	-	1.58
	H-Sr	56.38	40.16	3.45	-	1.49
	H-Ag	61.14	37.82	-	1.04	1.64
	H-Sr-Ag	56.39	39.41	3.34	0.86	1.54

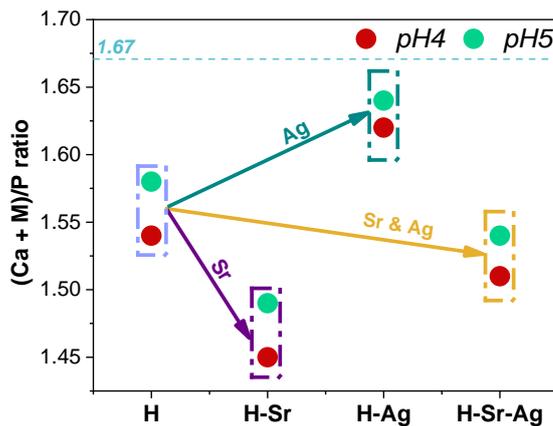


Figure 5.4. Evolution of (Ca+M)/P ratio, where M is Sr and/or Ag

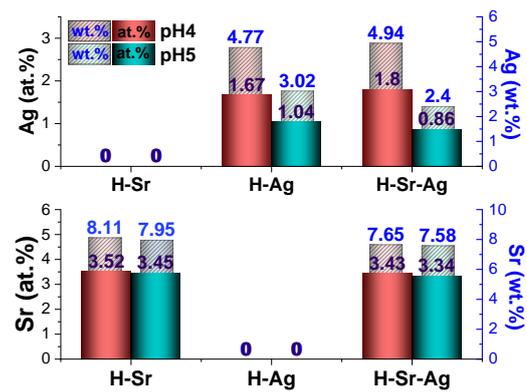


Figure 5.5. The concentration expressed in atomic and mass percentages of the 2 doping elements

Comparing the elemental composition registered for each doping element, it can be observed that the electrolyte's pH influences the amount found in the HAp coating, with notable differences being seen in the case of H-Ag coatings where the amount of Ag decreases from 1.67 at.% for those obtained at pH 4 to 1.04 at.% for those obtained at pH 5, while for the co-doped HAp coatings, H-Sr-Ag, the amount of Ag decreases from 1.80 at.% for those obtained

### 5.2.3. Phase composition of HAp-based coatings undoped and doped with Sr and/or Ag

Regardless of the electrolyte's pH value, the main phase identified was attributed to hydroxyapatite, according to ICDD standard #09-0432 (Figure 5.7). It was also noted the presence of a small diffraction peak, as a secondary phase, at  $2\theta = 30.31^\circ$  which according to ICDD standard #01-071-1759 can be assigned to monetite ( $\text{CaHPO}_4$ ).

The most intense diffraction peak is the one found at  $2\theta$  of  $\sim 26^\circ$  regardless the coating type and can be attributed to hydroxyapatite. This corresponds to (002) plane and indicates that the preferential orientation is perpendicular to the cp-Ti surface, along the  $c$  axis. This type of crystallization, along the  $c$  axis direction, supports cell proliferation thus favoring the osseointegration ability [243,244]. Also, addition of Sr and/or Ag in the HAp has led to shifts of the diffraction peak towards left (Figure 5.8).

In terms of the preferential degree of orientation (Figure 5.9), it is observed that the coatings obtained at a pH 5 present values of this parameter higher than those obtained at pH 4, being also closer to 1.73. However, one exception was noted in the case of H-Ag coatings, which showed a higher degree of the preferential orientation at pH 4 compared to that at pH 5.

Figure 5.7. XRD diffractograms of the HAp-based coatings at pH 4 and 5

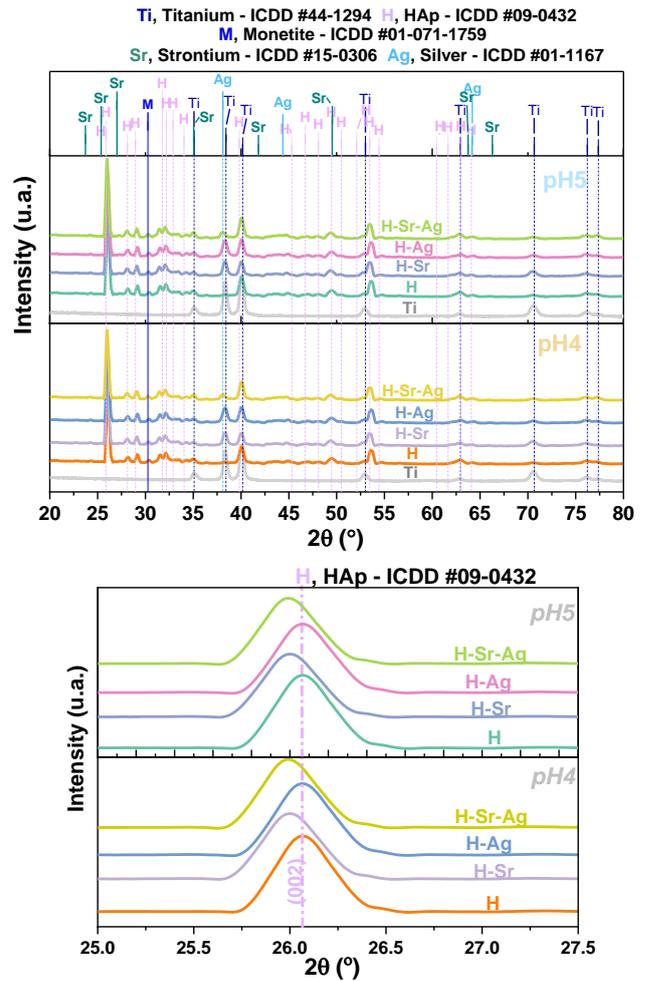


Figure 5.8. Section of interest of the diffractograms for the  $2\theta$  of  $\sim 26^\circ$  of the HAp coatings

Table 5.5. Crystallite size, lattice parameters, and crystallinity calculated for the HAp-based coatings

Samples and pH	Parameters	Diffraction peak for (002) reflection ( $^\circ$ )	Crystallite size d (nm)	Lattice Constants		Crystallinity (%)
				a=b ( $\text{\AA}$ )	c ( $\text{\AA}$ )	
HAp (#09-0432)		25.88	N/A	9.418	6.884	N/A
pH 4	H	26.09	26.04	9.418	6.861	46.55
	H-Sr	26.08	26.39	9.442	6.883	48.46
	H-Ag	26.03	26.65	9.423	6.872	49.93
	H-Sr-Ag	26.00	26.47	9.444	6.890	48.91
pH 5	H	26.07	26.11	9.423	6.865	46.92
	H-Sr	26.05	26.40	9.446	6.876	48.52
	H-Ag	26.01	26.31	9.426	6.867	48.05
	H-Sr-Ag	25.99	26.57	9.445	6.888	49.49

Considering the doping elements, the lattice parameters,  $a$  and  $c$ , have increased as a result of the difference between the ionic radius between the three doping elements [245]. Analyzing the influence of the electrolyte's  $pH$  on the coatings, it is observed that the use of a higher  $pH$  leads to the increment of the lattice constants (Table 5.5). The crystallinity and crystallite size have also increased by increasing the  $pH$ , suggesting that HAp-based coatings with higher crystallinity can be obtained at  $pH$  5.

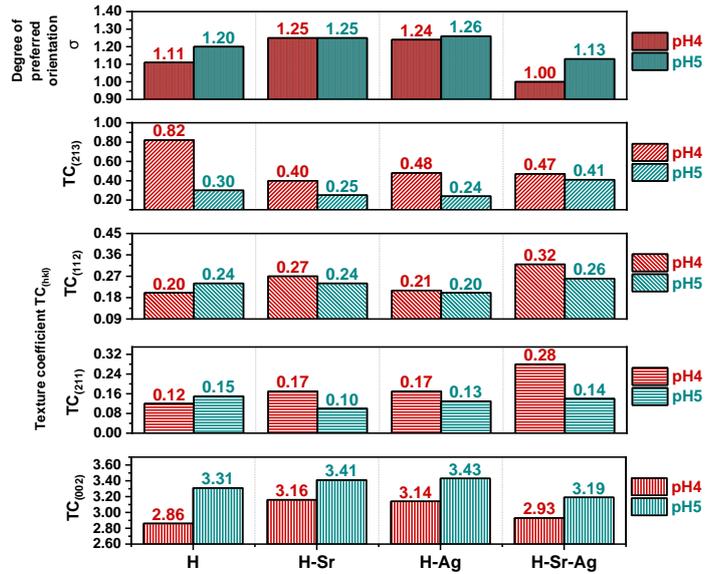


Figure 5.9. Texture coefficient and preferential degree of orientation

#### 5.2.4. Roughness and coatings thickness

Figure 5.13 shows the coating thicknesses and specific roughness parameters. The obtained results indicated that the roughness of the coatings is influenced by the electrolyte's  $pH$  and the doping elements. Therefore, changing the electrolyte's  $pH$  and the addition of the doping elements (Sr and/or Ag) has led to the development of uniform coatings with symmetrical topography and a roughness of 380-680 nm. Comparing the coating thicknesses for the undoped hydroxyapatite (H) as a function of the electrolyte's  $pH$  (Figure 5.13.) it can be seen that a higher  $pH$  favors the increment of the coating thickness.

This increase in thickness is due to the decrement in the amount of hydrogen released [165], resulting in a more compact and uniform coating. Compared to undoped HAp, addition of Sr or Ag into the HAp structure has also led to the increment of the coating thickness regardless of electrolyte's  $pH$ . Thus, it can be noted that in contrast to Sr, the registered values at  $pH$  5 indicate the development of a coating with a higher thickness than the one obtained for the undoped HAp, even though Ag tends to inhibit the layer growth. Regarding the coatings codoped with Sr and Ag, an increase of the coating thickness is observed for both  $pH$  values, with a higher value reached at  $pH$  4 than that obtained at  $pH$  5.

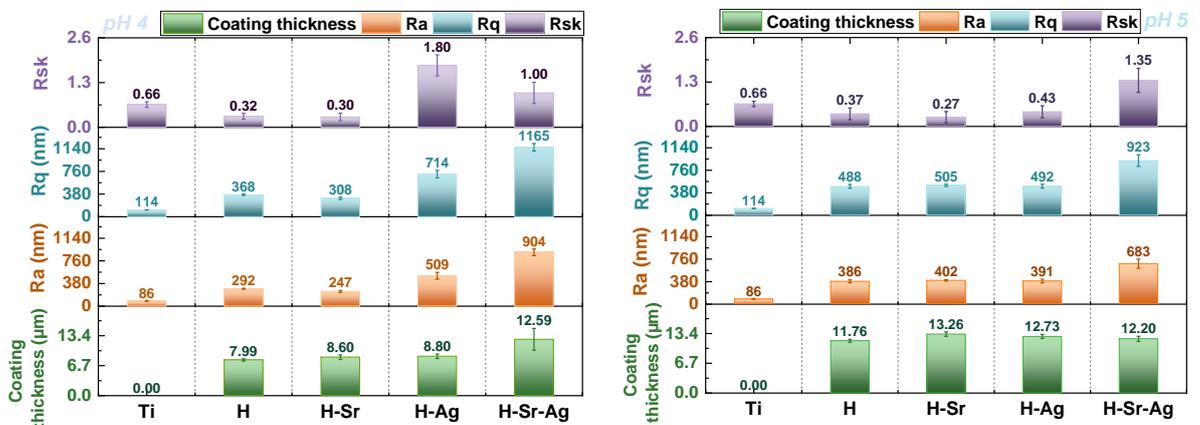


Figure 5.13. Coating thickness and roughness parameters of the HAp-based coatings

The deposition rate has increased by increasing the electrolyte's  $pH$  in all cases, less in the case of co-doped coatings with Sr and Ag, where it can be seen that the deposition rate did not show significant differences (Figure 5.14).

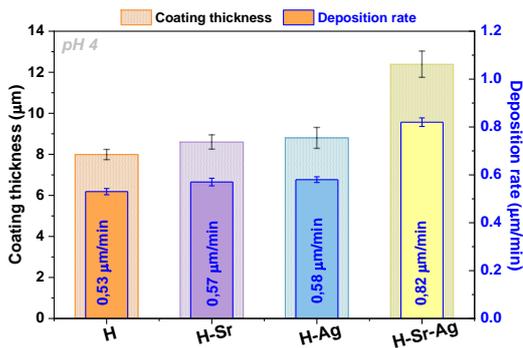


Figure 5.14. Deposition rate of HAp-based coatings obtained at pH 4

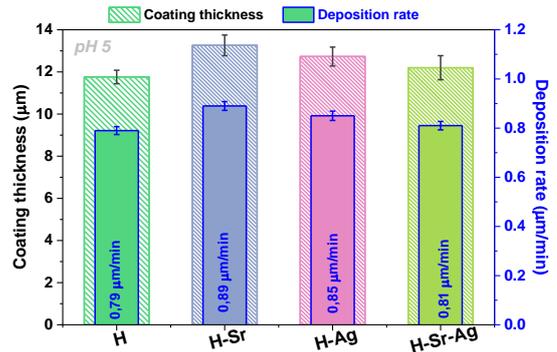


Figure 5.15. Deposition rate of HAp-based coatings obtained at pH 5

### 5.3. Conclusions

The characterizations presented in this chapter highlighted that the galvanostatic pulsed technique allows the development of uniform and compact hydroxyapatite-based coatings undoped and doped with strontium and/or silver on the surface of the metallic material exposed to the electrolyte, at both  $pH$  4 and  $pH$  5. One of the main electrochemical parameters involved in the deposition process is the electrolyte's  $pH$ . Therefore, in this chapter, the influence of the electrolyte's  $pH$  used in the electrochemical deposition on the properties and characteristics of the developed materials was evaluated. Following these results, the undoped and doped hydroxyapatite coatings with strontium and/or silver obtained at  $pH$  5 were selected due to their superior properties. Thus, in the following chapter, only these types of coatings will be further characterized and assessed.

## Chapter 6. Characterization and testing of HAp-based coatings obtained at pH 5

### 6.1. Chemical bonds and adhesion

#### 6.1.1. Chemical bonds

The Fourier transform infrared spectroscopy revealed the presence of phosphate groups ( $PO_4^{3-}$ ) in the range  $900 - 1118 \text{ cm}^{-1}$  and hydroxyl groups ( $OH^-$ ) in all hydroxyapatite-based coatings undoped and doped with Sr and/or Ag, obtained at  $pH$  5 on a pure titanium substrate, thus confirming the formation of the hydroxyapatite phase.

Also, the presence of the  $HPO_4^{2-}$  group in the FTIR spectra confirms that the coatings obtained by the galvanostatic pulsed technique at  $pH$  5 consists in calcium deficient HAp.

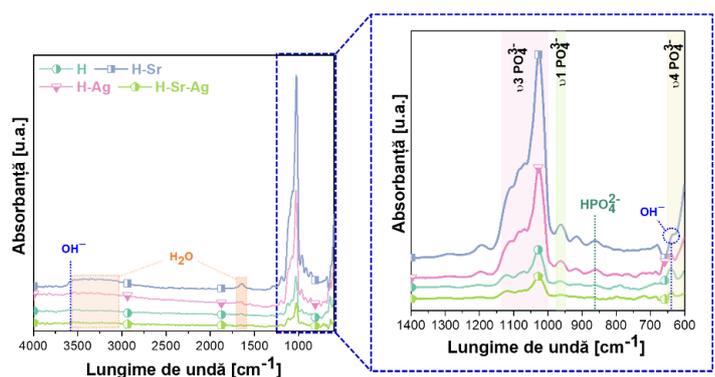


Figure 0.1. Chemical bonds of HAp-based coatings

### 6.1.2. Adhesion

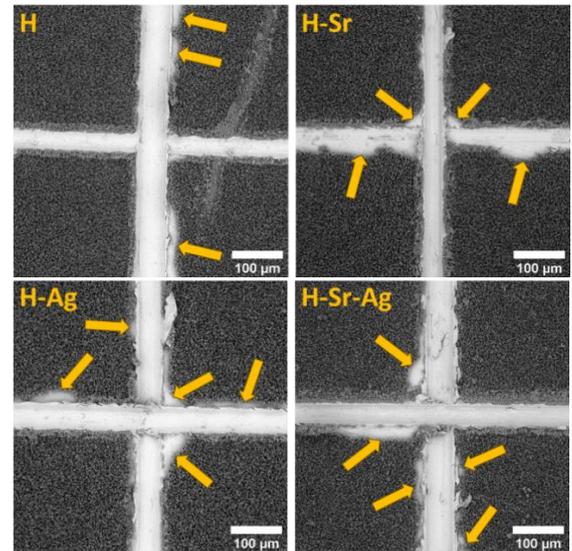
In Figure 6.3 are presented the SEM images of the imprints, following the adhesion assay by tape test according to the ASTM D 3359-17 standard [205]. From the SEM images it can be seen that the edges of the imprints are well defined, with very small, delaminated areas, due to the imprinting process.

Thus, a consistent and uniform coating of hydroxyapatite was observed on the substrate after the removal of the adhesive tape, and only a very thin, superficial coating on the active surface of the adhesive tape, was removed without affecting the structural integrity of the coating. All HAp coatings show good adhesion and can be classified into 4B category. Thus, the undoped HAp coating shows the lowest degree of delamination, followed by HAp coating doped, regardless of the nature of the element, where the affected areas have expanded. When doping with Sr, the degree of delamination is 3.81% while addition of Ag has led to an increment up to 4.27%. Even if both doping elements lead to a slight decrease of the HAp adhesion, the simultaneous addition of Sr and Ag does not have a concentrated negative effect on the adhesion and the delamination degree, even though these coatings have registered the highest value and the closest to the upper limit of the 4B category, but without exceeding 5%.

Thus, according to the results presented in Figure 6.4, obtained from the adhesion tests by scratch method, it can be observed that the highest value of the critical load is registered by the H coatings followed by the coatings doped with Ag and those co-doped with Sr and Ag, the latter ones being very close to each other.

After conducting the tests, the samples were analyzed in terms of elemental distribution at the end of the imprint to observe if there were any elements specific to the coatings, the results being presented in Figure 6.5.

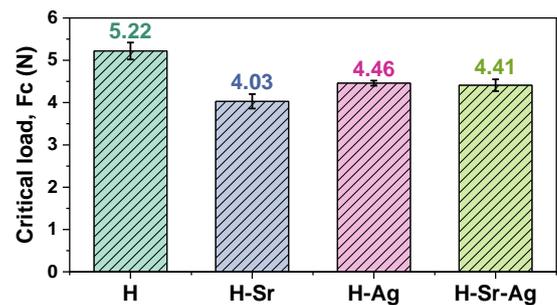
The results reveal that at the end of the imprint there are still traces of the main constituent elements of the hydroxyapatite-based coatings (Ca and P) but also of the doping elements, namely Sr and/or Ag.



**Figure 6.3.** SEM images of imprints after the adhesion test (orange arrows indicate areas with delaminated/peeling)

**Table 6.1.** Evaluation of the coating's adhesion according to ASTM D3359-17

	H	H-Sr	H-Ag	H-Sr-Ag
Detached surface (%)	1.55 %	3.81 %	4.57 %	4.86 %
Adhesion according ASTM D3359-17	4B	4B	4B	4B



**Figure 6.4.** Critical load ( $F_c$ ) obtained from scratch adhesion tests

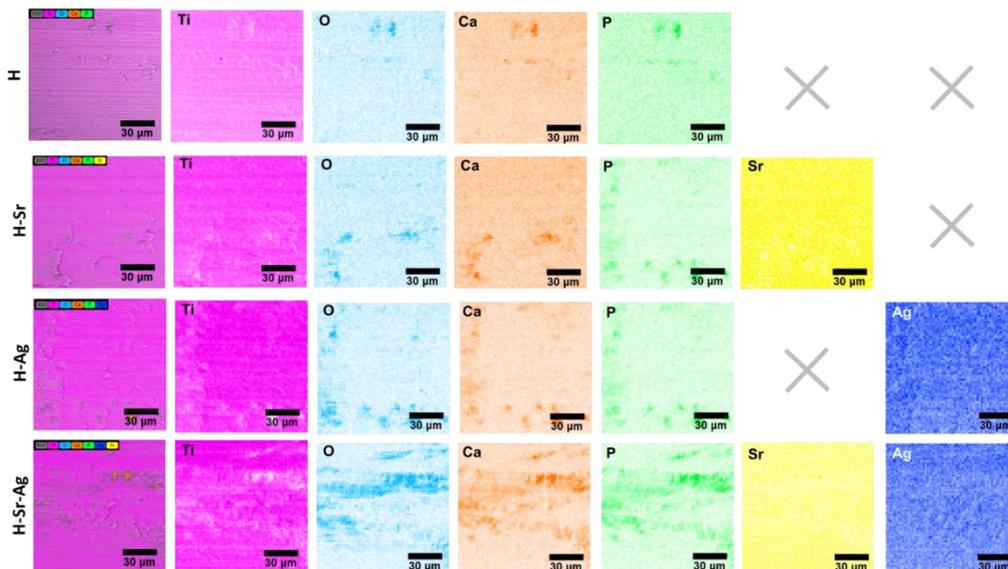


Figure 6.5. Elemental distribution within the scratches of all coatings

Figure 6.6. highlights that the two tests are in good agreement, as a higher critical force indicates better adhesion and later penetration of the coating, and a lower percentage of delaminated coating on the surface indicates a better adhesion of the coating.

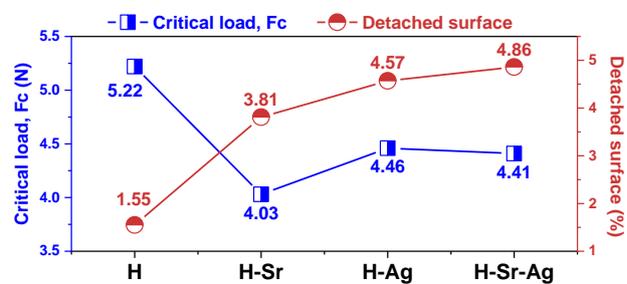


Figure 6.6. Evolution of adhesion results obtained by the two test methods

## 6.2. Wettability properties

### 6.2.1. Contact angle

Functionalization of the cp-Ti metallic substrate with HAp based coatings undoped and doped with Sr and/or Ag has led to the decrement of the contact angle measured with SBF, to values smaller than  $15^\circ$  (Figure 6.7), indicating a strong hydrophilic character of the HAp coatings, thus suggesting that the coating led to the modification of the surface chemistry.

Therefore, it can be stated that the HAp-based coatings show a strong hydrophilic character.

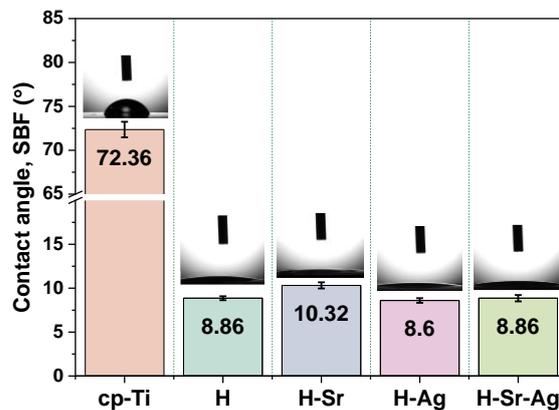


Figure 6.7. The contact angle of the surfaces tested with SBF

### 6.2.2. Surface free energy

The contact angle results for distilled water, ethylene glycol and toluene used to obtain the surface free energy of the HAp-based coatings are found in Figure 6.8. They indicated that irrespective of the liquid used, all coatings have a contact angle smaller than  $90^\circ$  denoting a hydrophilic character. In Figure 6.9 are presented the obtained surface free energy values.

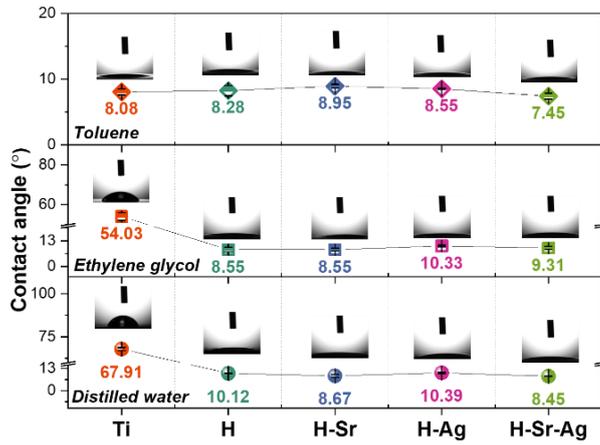


Figure 6.8. Contact angle of HAp-based surfaces measured with distilled water, ethylene glycol and toluene

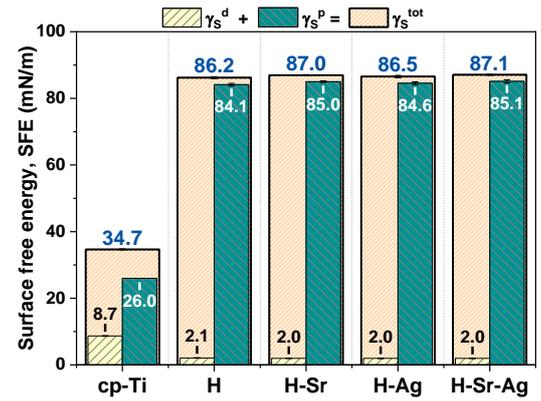


Figure 6.9. Surface free energy for the investigated surfaces

The HAp-based coatings have registered contact angle values between 7.5° and 10.5°, being much smaller than the ones obtained by the cp-Ti substrate. The H-Sr-Ag coating have registered the highest value of the  $\gamma_s^{tot}$  of 87.1 mN/m, closely followed by the H-Sr coating with a  $\gamma_s^{tot}$  of 87.0 mN/m, H-Ag which have reached a value of 86.6 mN/m and H with a  $\gamma_s^{tot}$  of 86.2 mN/m.

### 6.3. Electrochemical behavior

Figure 6.11. shows the evolution of the open circuit potential, and Figure 6.12. presents the Tafel plots for the cp-Ti and the HAp based coatings undoped and doped Sr and/or Ag, while the electrochemical parameters extracted from the obtained results can be found in Table 6.3.

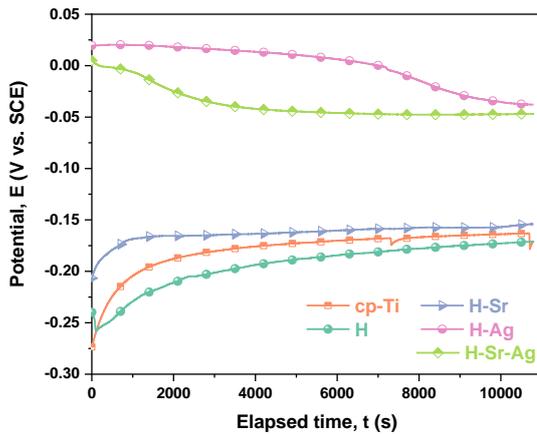


Figure 6.11. Evolution of open circuit potential for cp-Ti and the HAp-based coatings

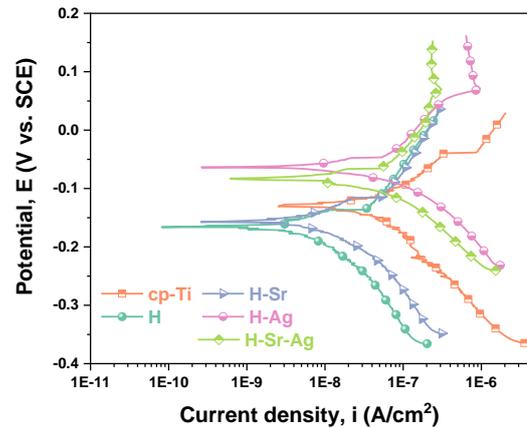


Figure 6.12. Evolution of Tafel plots for cp-Ti and the HAp-based coatings

It is known that a material has a good electrochemical behavior when the values of the open circuit potential ( $E_{OC}$ ) and the corrosion potential ( $E_{corr}$ ) are more electropositive, the corrosion current density ( $i_{corr}$ ) has smaller values, and the polarization resistance ( $R_p$ ) have high values [232,233]. Considering these criteria and evaluating the results obtained from the electrochemical tests, it was observed that the corrosion resistance of the pure titanium substrate was improved by coating it with HAp-based coatings.

Table 0.1. Electrochemical parameters for cp-Ti and the HAp-based coatings

Samples	$E_{OC}$ (mV)	$E_{corr}$ (mV)	$i_{corr}$ (nA/cm²)	$\beta_c$ (mV)	$\beta_a$ (mV)	$R_p$ (kΩ × cm²)	$Pe$ (%)
cp-Ti	-170.38	-129.79	83.54	189.97	123.87	390.24	-
H	-171.07	-166.24	18.07	220.93	136.73	2032.17	78.4
H-Sr	-154.12	-161.07	26.27	196.54	146.89	1391.28	68.5
H-Ag	-37.88	-63.20	52.63	103.21	165.55	525.20	37.0
H-Sr-Ag	-46.93	-84.42	48.63	120.37	200.22	672.12	41.8

The undoped HAp-based coatings showed the lowest  $i_{corr}$ , and the highest  $R_p$  and protective efficiency ( $P_e$ ). The values recorded by H-Sr were close to those obtained by the H coating. Although the addition of Ag in the HAp structure has led to a weaker electrochemical behavior compared to the other types of coatings, it cannot be said that the properties are poor due to the metallic character of Ag, thus indicating that it has not been fully substituted.

#### 6.4. Evaluation of *in vitro* behavior in SBF and PBS

##### 6.4.1. Biomineralization assays in SBF

Figure 6.14. shows the mass evolution of the newly formed apatite following the immersion tests in SBF of the cp-Ti substrate and the HAp-based coatings undoped and doped with Sr and/or Ag. According to the results, the HAp-based coatings showed an increment of mass for all time intervals compared to the substrate. Among these, the co-doped coatings stand out as it recorded the highest increase at the end of the tests, of 16.43 mg (21 days). However, it is observed that regardless the immersion period in SBF, the amount of newly formed apatite on the coatings surface is progressively increasing after each time interval, indicating that the coating favor the formation of a new layer of apatite, and thus have enhanced the osseointegration ability [268].

##### 6.4.2. Biodegradation assays in PBS

Figure 6.16. shows the mass evolution of the cp-Ti substrate and HAp-based coatings exposed to PBS. It can be observed in comparison to the undoped hydroxyapatite, the doped hydroxyapatite-based coatings have registered higher mass losses during the entire testing period, thus highlighting the beneficial effect of the doping elements on the degradation capacity of hydroxyapatite.

The smallest mass loss of  $-0.87$  mg was recorded by the H coating, followed by H-Sr with  $-1.05$  mg and H-Ag with  $-1.08$  mg. The Ag and Sr co-doped coating showed the highest mass loss of  $-1.12$  mg. This degradation rate in PBS could be attributed to the presence of an amorphous phase in the HAp structure [117] which favors its degradation.

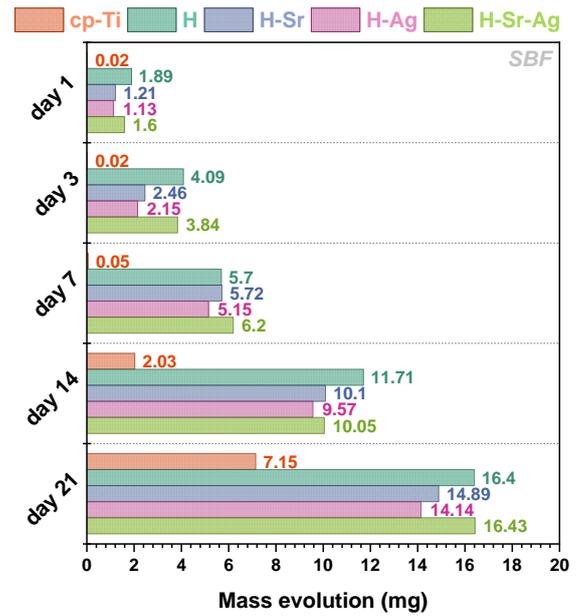


Figure 6.14 The mass evolution for cp-Ti and HAp-based coatings exposed in SBF

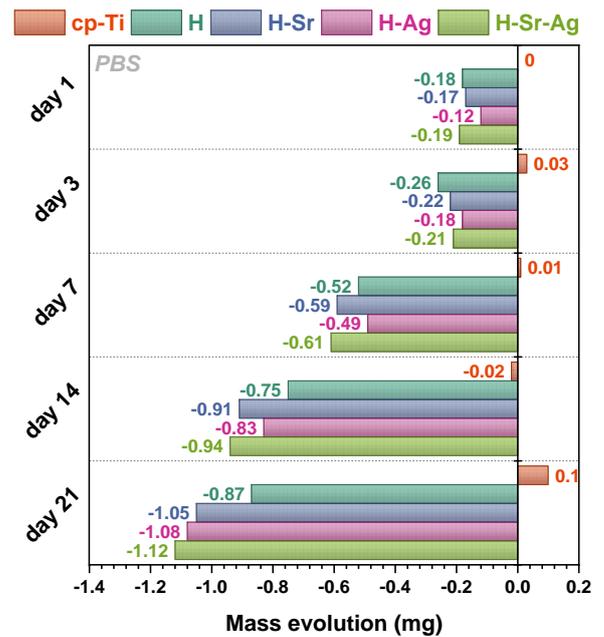


Figure 6.16 The mass evolution for cp-Ti and HAp-based coatings exposed to PBS

## 6.5. Cell assays

### 6.5.1 MC3T3-E1 Cell Viability and Proliferation

Figure 6.17. shows the fluorescence microscopy images of MC3T3-E1 preosteoblasts grown in direct contact with the cp-Ti and the HAp coatings for 1 and 3 days, where live cells are marked in green and the dead cells in red. According to Figure 6.17, the preosteoblasts seeded on the investigated surfaces proliferated from 1 to 3 days, changing their morphology to an elongated shape. Compared to the HAp-based coatings, the cp-Ti substrate showed the highest density increment at both 1 and 3 days of incubation. Dead cells, highlighted by white arrows in Figure 6.17, were observed only in the case of Ag-doped HAp coatings, emphasizing that it can induce a slight cytotoxic effect, most likely due to the concentration of Ag in the coatings and/or due to the localized concentration of Ag according to SEM images.

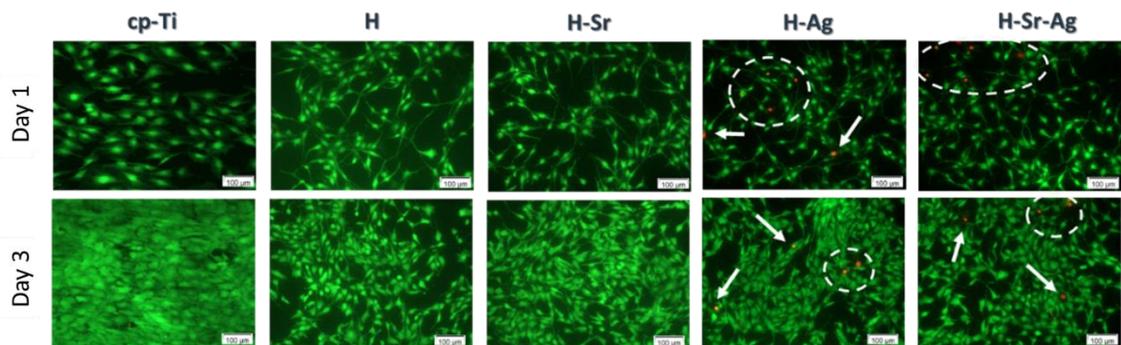


Figure 6.17. Fluorescence microscopy images of MC3T3-E1 preosteoblasts grown in direct contact with the cp-Ti substrate and the developed coatings for 1 and 3 days

Figure 6.18. presents the results obtained from the CCK-8 test, from which it can be seen that after one day of maintenance in the culture medium, regardless the coating type, with or without the doping element, there are no major differences, registering very close values. Differences in cell proliferation were reached after 3 days of maintaining the substrate and HAp-based coatings in the culture medium, where higher optical density (OD) values are noted. H-Sr coatings showed an increase in the density of preosteoblasts, after 3 days of incubation, compared to H and H-Ag coatings, and an increase in DO values was also noted.

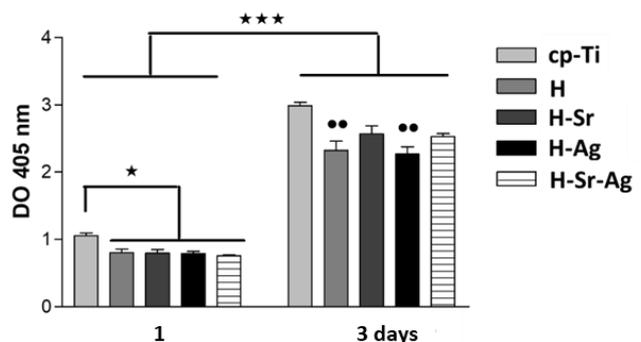


Figure 6.18. CCK-8 assay showing the proliferative capacity of cells grown for 1 and 3 days in contact with the developed HAp-based coatings and the bare cp-Ti (\*\*\*)  $p < 0.001$  vs. 1 day; \*  $p < 0.05$  for H vs. Ti coatings at 1 day; \*\*  $p < 0.01$  for H and H-Sr vs. Ti at 3 days)

### 6.5.2. MC3T3-E1 preosteoblasts cell morphology

Figure 6.19 highlights on the surface of the cp-Ti substrate, along the cell body, a polygonal-elongated morphology showing stress fibers. Compared to the cp-Ti substrate, preosteoblasts grown for one day on the surfaces of the HAp-based coatings show a dendritic, filiform morphology, while after 3 days, the preosteoblasts show a cuboidal morphology. It is noteworthy that the preosteoblasts grown on HAp-based coatings show intercellular contacts through fine cytoplasmic extensions.

The SEM images of MC3T3-E1 preosteoblasts (Figure 6.20 and 6.21) revealed that cells adhered and spread on the investigated surface showing different morphological features and plasma membrane protrusions in the form of lamellipodia (sheet-like extensions) and filopodia

(digitiform-like extensions). Thus, the preosteoblasts found on the surface of the substrate adopt an elongated polygonal conformation predominantly with lamellipodia around the cell body, while on the HAp-based coatings the cells show several conformations, respectively fusiform and polygonal-elongate with numerous lamellipodia especially after 1 day of incubation but also filopodia which are more frequent after 3 days of growth in culture medium.

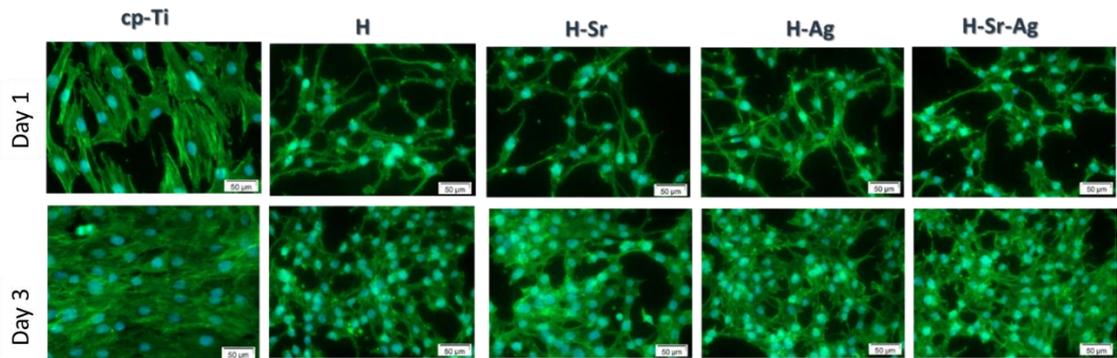


Figure 6.19. 10. Morphology of MC3T3-E1 preosteoblasts grown in direct contact with HAp-based coating and cp-Ti

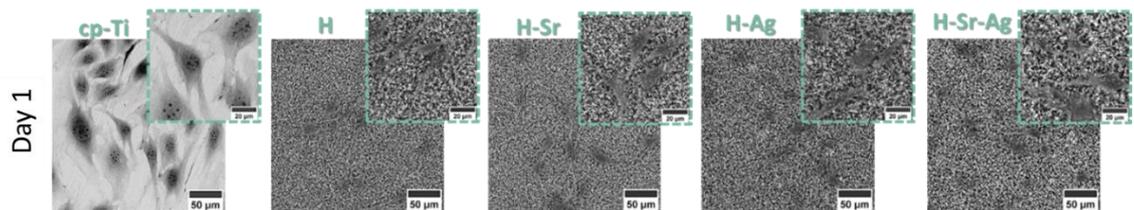


Figure 6.20. SEM images of MC3T3-E1 preosteoblasts grown in direct contact with HAp-based coating and cp-Ti after 1 day

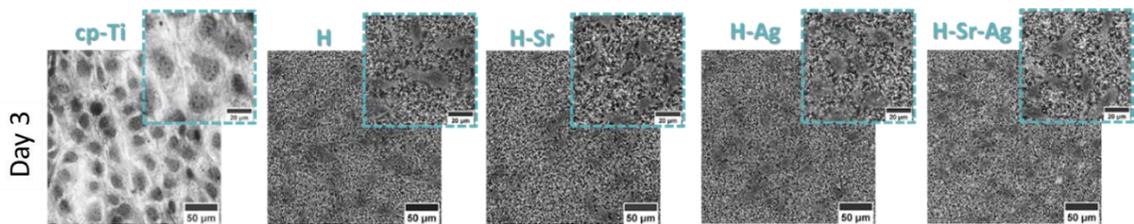


Figure 6.21. SEM images of MC3T3-E1 preosteoblasts grown in direct contact with HAp-based coating and cp-Ti after 3 days

### 6.5.3. MC3T3-E1 cell differentiation

According to the results presented in Figure 6.22. it can be seen that after 7 days, there are no significant differences between the coatings and the cp-Ti substrate. The ALP activity of preosteoblasts has increased from 7 to 14 days indicating that the osteogenic differentiation process is favored.

The collagen level revealed no significant differences between the cp-Ti substrate and the undoped HAp-based coatings at both 2 and 4 weeks, respectively. Similar to ALP, Sr favors preosteoblast differentiation whether used as a single element or together with Ag.

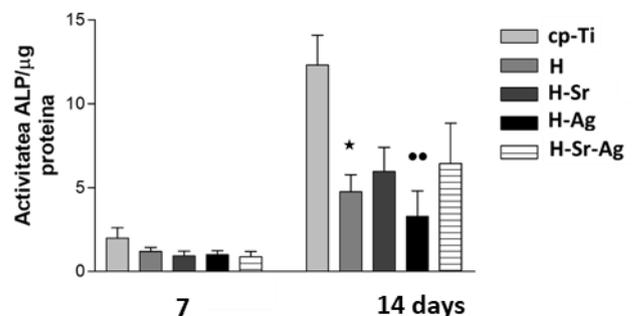


Figure 6.22. Enzyme activity of the intracellular ALP at 7 and 14 days after seeding (\*  $p < 0.05$  for H-Sr vs. Ti sample and \*\*  $p < 0.01$  for H-Ag vs. Ti at 14 days);

Figure 6.24. shows the quantification of ECM mineralization determined by highlighting the calcium deposit. Compared to the cp-Ti substrate, all HAp-based coatings registered higher DO values. It is noted that H and H-Sr-Ag coatings have obtained the highest DO values followed by H-Sr and H-Ag coating.

### 6.6. Conclusion

The galvanostatic pulsed deposition is a technique that allows the development of undoped and strontium and/or silver doped hydroxyapatite coatings on the surface of the metallic material exposed to the electrolyte at pH 5.

Following the results obtained, it is noted that the doping elements influence the properties of the hydroxyapatite-based coatings.

However, the properties of hydroxyapatite-based coatings can be modulated by changing the parameters involved in the deposition process, in order to obtain coatings with superior properties that can be used in orthopedic and dental applications.

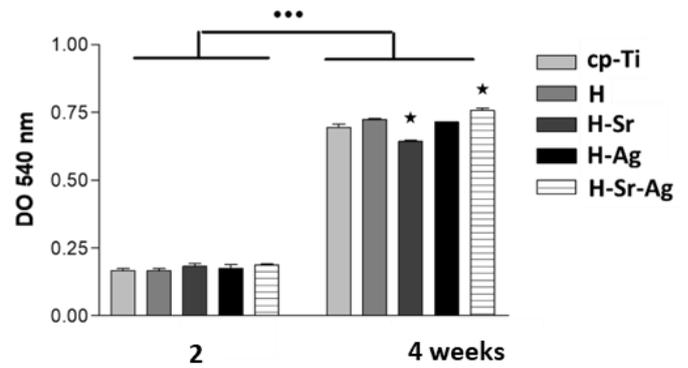


Figure 6.23. The level of collagen synthesis and extracellular matrix deposition after 3 weeks of culture (●●●  $p < 0.001$  vs. 2 weeks; \*  $p < 0.05$  for H-Sr and H-Sr-Ag vs. Ti at 4 weeks)

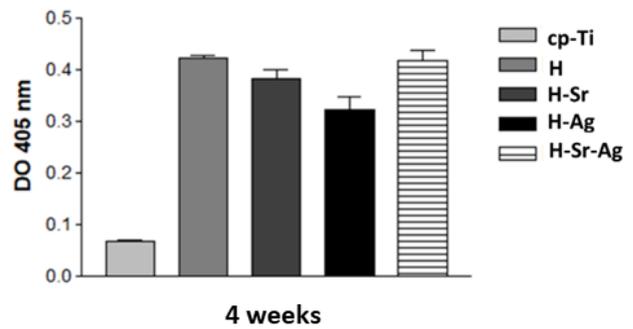


Figure 6.24. The level of matrix mineralization as quantified by Alizarin Red staining at 4 weeks post-seeding (\*\*\*)  $p < 0.001$  for H-Sr and H-Sr-Ag vs. Ti, \*\*  $p < 0.01$  for H-Ag vs. Ti

## Conclusions, personal contributions and future research directions

The main objective of current research carried out on the biofunctionalization of implantable medical devices is to reduce the recovery time of patients undergoing surgery by accelerating osseointegration and decreasing the risk of failure associated with intrahospital infections. Although biomaterials pass from one generation to another by accumulating superior properties, some clinical factors that can affect their success *in vivo* are still present.

The PhD thesis "*Studies and research on hydroxyapatite-based coatings development with high osseointegration properties and antibacterial ability using electrochemical techniques*" represents an extensive up-to-date study which aim to solve some clinical problems by increasing the osseointegration capacity of metallic implants biofunctionalized with biomaterials that have osteoconductive and antibacterial properties by modern techniques.

The studies, and experimental research carried out, have led to the following general conclusions:

- the hydroxyapatite coating undoped and doped with Sr and/or Ag were obtained through two electrochemical techniques, namely the potentiostatic pulsed technique and the galvanostatic pulsed technique;
- in the case of both techniques, hydroxyapatite-based coatings with a Ca/P ratio close to the stoichiometric one of 1.67 were obtained; since the values reached were slightly smaller, it can be said that the obtained hydroxyapatite is deficient in Ca.

### The potentiostatic electrochemical technique in pulses:

- in order to obtain coatings (undoped and Ag-doped hydroxyapatite) of similar thicknesses ( $\sim 7 \mu\text{m}$ ) the electrochemical parameters were optimized by changing the electrochemical parameters (deposition time, applied deposition potential, concentration of ions in the electrolyte);
- the morphology of hydroxyapatite-based coatings consists of a plate-like crystals, with a preferential orientation, perpendicular to the substrate surface;
- addition of Ag into the hydroxyapatite, has induced some changes to the coatings morphology, since the plate-like crystals become wider and thinner, and at the same time areas with small agglomerations spread within plate-like network, attributed to Ag, were also identified;
- the elemental composition revealed that after the addition of Ag, the Ca/P ratio has increased from a value of 1.58 to 1.62, the latter one being closer to that of stoichiometric hydroxyapatite (Ca/P=1.67);
- the phase composition have shown that both coatings presented diffraction peaks specific to hydroxyapatite, and addition of Ag has led to shifts towards smaller angles of the diffraction peaks, highlighting the fact that the  $\text{Ag}^+$  ions are partially replacing the  $\text{Ca}^{2+}$  ions found in the hydroxyapatite structure; the value of the lattice constants  $a=b$  have increased after the addition of Ag ions, indicating that the crystallographic cell of hydroxyapatite was modified, also confirming the substitution of  $\text{Ca}^{2+}$  with those of the doping element; the crystallite size decreased from a value of 35.88 nm obtained for undoped hydroxyapatite to a value of 21.23 nm reached for the Ag-doped hydroxyapatite; a similar trend was also noted for the crystallinity degree, which has decreased from a value of 29.07 % obtained for the undoped hydroxyapatite to a value of 22.50 % for the hydroxyapatite doped with Ag;
- the roughness of the hydroxyapatite coatings has increased considerably after the addition of  $\text{Ag}^+$  ions, as highlighted by the Ra roughness parameter, which has increased by approximately 5 times;
- the biomineralization tests revealed that compared to the substrate, both types of coatings have improved this characteristic, while that presence of  $\text{Ag}^+$  ions have further enhanced the hydroxyapatite biomineralization capacity;

- regarding the electrochemical behavior, both types of coatings have improved the corrosion resistance of Ti6Al4V substrate, in both simulated biological solution and in artificial saliva, suggesting that the proposed coatings can be used in both medical applications, namely orthopedic and dental field.

#### **The galvanostatic electrochemical technique in pulses:**

- the deposition parameters were selected so that: (i) all designed coatings (hydroxyapatite undoped and doped with Sr and/or Ag) to be obtained at the same values of these parameters; (ii) the actual deposition time to be smaller in comparison with the one used in the electrochemical potentiostatic technique, aiming to optimize the deposition time and the energy efficiency at the same time;
- the hydroxyapatite-based coatings undoped and doped with Sr and/or Ag were obtained by using electrolytes with similar chemical compositions but with different *pH* values of 4 and 5, respectively.;
- the obtained morphology revealed very thin, long and wide ribbon-like crystal that undergo small changes in accordance with the *pH* value and the nature of the doping element; increasing the *pH* value of the electrolyte from 4 to 5 leads to slightly narrower ribbons, regardless of the doping element used; the addition of silver in the hydroxyapatite structure has led to a morphology consisting of ribbon-like crystals and flower-like agglomerations, regardless of the *pH* value of the electrolyte; however, it was noted that when the *pH* value was adjusted to 5, the density of the flower-like agglomerations in the Ag-containing coatings has decreased, suggesting that the usage of a higher *pH* value leads to a better dispersion of the Ag within the coatings;
- the elemental composition confirmed the presence of hydroxyapatite-specific elements (calcium and phosphorus) and the doping elements (silver and strontium); the (Ca+M)/P ratio, where M= Sr, Ag, Sr+Ag, registered values between 1.45 and 1.64 irrespective of the electrolyte's *pH* or the doping element used, with higher values being reached for the coatings deposited at *pH* 5; by increasing the electrolyte's *pH*, the amount of strontium within the coatings was not majorly influenced, in comparison with Ag<sup>+</sup>, where a dependence between the electrolyte's *pH* and the amount of Ag subsequently found in the coatings (increment of the *pH* leads to the decrement of the Ag amount) was observed; the coatings co-doped with strontium and silver ions show smaller amounts of silver at *pH* 5, indicating that strontium inhibits silver substitution by increasing the electrolyte's *pH*;
- the X-ray diffraction analysis has highlighted that irrespective of the electrolyte's *pH* and the nature of the doping element, the main phase identified is hydroxyapatite, and also a secondary phase of monetite (precursor of hydroxyapatite); no major differences were noted between the diffractograms of the coatings obtained at *pH* 4 and 5, apart from slightly higher intensities of those obtained at *pH* 5; the diffraction peak found at  $2\theta$  of  $\sim 26^\circ$  and associated with the (002) plane shows the highest intensity regardless of the type of doping element used or the electrolyte's *pH* value, indicating that the preferential orientation is along the *c* axis; the X-ray diffractograms revealed a slight shifts towards lower angles of the diffraction peak found at  $2\theta$  of  $\sim 26^\circ$  after the addition of the doping elements indicating that the structure of the hydroxyapatite underwent changes, thus confirming the doping; from the point of view of structural parameters, it was noted that the lattice parameters *a* and *c* of the hydroxyapatite have increased with the addition of the doping elements, also confirming that the doping elements have successfully substituted part of the Ca atoms in the hydroxyapatite structure; from the point of view of crystallinity, it was noted that the addition of Sr and/or Ag doping elements in the HAp structure have led to the increment of this parameter, regardless of the *pH* value of the electrolyte (the minimum degree of crystallinity reached for the undoped hydroxyapatite obtained at *pH* 4, was of 46.64 %);

- the roughness of the coatings, according to the Ra parameter, increases in the case of coatings obtained at pH 5, except for the coatings doped or co-doped with Ag; the highest roughness was recorded for the hydroxyapatite coatings co-doped with Sr and Ag ions obtained in the case of pH 4;
- the coating thickness had closer values when using the electrolyte with pH 5 (11.5 and 13.5  $\mu\text{m}$ ), while the coatings obtained at pH 4 have registered values between 8 and 12.6  $\mu\text{m}$ ; the use of a more basic electrolyte have led to the increment of the coatings thickness regardless the nature of the doping element, with the exception of the hydroxyapatite coatings co-doped with Sr and Ag where the coating thickness was not strongly influenced by the pH value (the difference being of only 0, 4  $\mu\text{m}$ );
- the deposition rate has increased by increasing the pH value, indicating that it directly influences the growth and nucleation process of hydroxyapatite;

#### **HAp coatings undoped and doped with Sr and/or Ag obtained in electrolyte with pH 5:**

- Fourier transform infrared spectroscopy revealed the presence of phosphate ( $\text{PO}_4^{3-}$ ) and hydroxyl ( $\text{OH}^-$ ) groups in all coatings, thus confirming the presence of the hydroxyapatite phase;
- the adhesion tests by using the "tape test" method showed that the hydroxyapatite-based coatings obtained at pH 5 have a good adhesion to the substrate, being classified in category 4B according to the ASTM D3359-09 standard; even if the delaminated surfaces had values smaller than 5%, the undoped hydroxyapatite coatings are noteworthy since the delaminated surface was of only 1.55%;
- the adhesion tests obtained by the scratch method confirmed that the undoped hydroxyapatite-based coatings have a better adhesion to the substrate, registering the highest critical load, of 5.22 N, followed by the coatings doped with Sr and/or Ag which have recorded appropriate values, within the range of 4.03 N – 4.46 N;
- considering the two types of tests used to evaluate the adhesion, it can be said that the addition of Sr and/or Ag doping elements in the HAp structure have led to a slight decrement of the coatings adhesion;
- in terms of wettability, the contact angle analysis with simulated body fluid (SBF) highlighted that all coatings have a strong hydrophilic character, registering contact angles in the range of  $8^\circ$  -  $10^\circ$ ;
- the surface free energy of the substrate was significantly improved by covering it with hydroxyapatite-based coatings, the values recorded in their case being approximately 2.5 times higher;
- the hydroxyapatite coatings undoped and doped with Sr and/or Ag have led to the improvement of the electrochemical behavior of the pure titanium substrate in SBF; the best electrochemical behavior highlighted by the lowest corrosion current density, the highest polarization resistance and the best protection efficiency against the corrosive attack was registered by undoped HAp-based coatings;
- the biomineralization tests in SBF showed that all coatings favor the precipitation of a new layer of apatite on their surface after 21 days of immersion in the testing media, thus improving the biomineralization capacity of titanium; the best results in terms of newly formed apatite mass gain on the surface were registered for the HAp based coatings co-doped with Sr and Ag;
- the biodegradation tests carried out in phosphate buffer solution (PBS) have shown that the doped HAp accelerates the degradation of the hydroxyapatite-based coatings; after 21 days of immersion, the HAp-based coatings co-doped with Sr and Ag showed the highest mass loss;
- the studies regarding the response of the preosteoblasts on the hydroxyapatite coatings undoped and doped Sr and/or Ag indicated that these surfaces favor the adhesion and proliferation capacity of preosteoblasts, while also favoring the osteogenic differentiation of preosteoblasts;

- the addition of Sr into the hydroxyapatite-based coatings exerted beneficial effects on preosteoblast response, regardless of whether the coating contained Ag or not.

### Personal contributions

The original contributions of this PhD thesis present novel elements in materials engineering with applications in medicine. These contributions can be summarized as follows:

- ✓ Performing a literature overview focused on the most used materials for implantable medical devices manufacturing along with the deposition methods used to obtain hydroxyapatite coatings undoped or doped with different ions, in order to obtain new surface features necessary from a clinical point of view;
- ✓ Selection of the co-doping elements to meet the current clinical requirements, especially improving the osseointegration properties and inducing antibacterial ones;
- ✓ Identification and selection of the hydroxyapatite deposition methods that can offer the possibility of both doping and co-doping by using the same deposition parameters;
- ✓ Selection and optimization of the nature and values of the deposition parameters so that they can be used in order to obtain all designed materials;
- ✓ Development of hydroxyapatite based coatings undoped and doped with Ag ion by the potentiostatic method in pulses with similar coating thickness by changing the deposition parameters, and their complex, comparative and detailed characterization in terms of morphology, elemental composition, phase composition, bonds chemical properties, structural parameter values, crystallinity, crystallite size, roughness, biomineralization capacity and electrochemical behavior in simulated body fluid (SBF) and artificial saliva;
- ✓ Development for the first time of hydroxyapatite-based coatings co-doped with Sr and Ag through the galvanostatic electrochemical method in pulses with a ribbon-like morphology;
- ✓ Complex, comparative and detailed characterization of the undoped and Sr- and/or Ag-doped hydroxyapatite coatings obtained at different electrolyte's *pH* values, in terms of morphology, elemental composition, phase composition, structural parameters values, crystallinity, crystallite size, roughness, coating thickness and deposition rate;
- ✓ Complex *in vitro* testing of the hydroxyapatite based coatings co-doped with Sr and Ag obtained by the galvanostatic pulsed electrochemical method in an electrolyte with a *pH* 5 in terms of:
  - electrochemical behavior in simulated body fluid (SBF),
  - bioactivity (biomineralization in SBF and biodegradation in phosphate buffer solution - PBS)
  - cellular response on preosteoblast cell cultures (viability, proliferation, morphology and differentiation);
- ✓ Development of hydroxyapatite-based coatings co-doped with Sr and Ag in optimal amounts to support the adhesion and proliferation capacity of preosteoblasts and at the same time the osteogenic differentiation of preosteoblasts.

### Future research directions

The studies and research carried out in this PhD thesis have led to the understanding of some physico-chemical processes that underlie the nucleation and growth of the hydroxyapatite coatings undoped and doped with Sr and/or Ag ions, but also their influence on the required clinical characteristics. Nonetheless, some issues addressed in this study remain open to be deepened by extensive characterization and testing of the hydroxyapatite coatings undoped and Sr- and/or Ag-doped as follows:

1. Evaluation of the electrochemical behavior in other acellular synthetic biological media (different types of artificial saliva, Dulbecco's media) in order to understand this behavior in the case of implantable medical devices coated with hydroxyapatite;

2. Quantification of the amount of ions released on the short and long term following electrochemical tests in various synthetic biological fluids, but also following bioactivity tests;
3. Coatings investigation through micro-CT techniques to determine the degree of porosity and understand the growth mechanism of the ribbon-like crystals;
4. Continuing the *in vitro* study regarding the proliferation and adhesion capacity of preosteoblasts over longer periods of time and by using human fibroblast cell line;
5. Deepening the osteogenic differentiation studies by quantifying proteins (eg: osteopontin, osteocalcin) that favors the bone remodeling process;
6. Performing antibacterial tests on bacteria specific to infections associated with implantable devices operations such as Gram-positive (*S. aureus*) and Gram-negative (*E.coli*) to quantify the antibacterial and antifungal properties;
7. Evaluation of the hydroxyapatite-based coatings biocompatibility properties by carrying out *in vivo* assays on animal models.

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## List of scientific papers published

During the PhD program, a number of 8 scientific papers were published in ISI ranked Journals and 24 papers were presented at international conferences. All these scientific papers and conference papers are within the field of the PhD thesis.

### Scientific papers published in ranked ISI journals

1. **Ungureanu Elena**, Vladescu (Dragomir) A., Parau A.C., Mitran V., Cimpean A., Tarcolea M., Vranceanu D.M., Cotrut M.C., [In Vitro Evaluation of Ag- and Sr-Doped Hydroxyapatite Coatings for Medical Applications](#), *Materials*, **2023**, Vol. 16, Iss. 15, Art.no. 5428, pp.28, DOI: 10.3390/ma16155428, FI=3,400, **cu rezultate obtinute din prezenta Tezã de doctorat**;
2. Cotrut M.C., **Ungureanu Elena**, Ionescu I.C., Zamfir R.I., Kiss A.E., Parau A.C., Vladescu A., Vranceanu D.M., Saceleanu A., [Influence of Magnesium Content on the Physico-Chemical Properties of Hydroxyapatite Electrochemically Deposited on a Nanostructured Titanium Surface](#), *Coatings*, **2022**, Vol. 12, Iss. 8, Art.no. 1097, pp. 25, DOI: 10.3390/coatings12081097, WOS:000846987400001, FI= 3,236;
3. Vranceanu D.M., **Ungureanu Elena**, Ionescu I.C., Parau A.C., Kiss A.E., Vladescu A., Cotrut M.C., [Electrochemical Surface Biofunctionalization of Titanium through Growth of TiO<sub>2</sub> Nanotubes and Deposition of Zn Doped Hydroxyapatite](#), *Coatings*, **2022**, Vol.12, Iss. 1, Art.No. 69, pp. 16, DOI: 10.3390/coatings12010069, WOS:000765085600001, FI=2,881;
4. **Ungureanu Elena**, Vranceanu D.M., Vladescu A., Parau A.C., Tarcolea M., Cotrut M.C., [Effect of doping element and electrolyte's ph on the properties of hydroxyapatite coatings obtained by pulsed galvanostatic technique](#), *Coatings*, **2021**, Vol 11, Nr. 12, Art.no. 1522, pp. 15, DOI: 10.3390/coatings11121522, WOS:000736213900001, FI=2,881, **cu rezultate obtinute din prezenta Tezã de doctorat**;
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- 10 Pana, A.C. Parau, **E. Ungureanu**, J. Raczowska, C. Vitelaru, M. Dinu, K. Awsiu, D.M. Vranceanu, C.M. Cotrut, and A. Vladescu- *ZrCu-based thin films metallic glasses with addition of Ca, Mo, Sr, Mg and Si*, 9th International Conference on Materials Science and Technologies – RoMat 2022, 24-25 November **2022**, Bucharet, Romania
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