Digest Journal of Nanomaterials and Biostructures Vol. 9, No. 2, April – June 2014, p. 575 - 584

# ELABORATION AND CHARACTERIZATION OF THE ELECTRODEPOSITED PHOSPHATES MASSES DOPED WITH VARIOUS IONS ON STAINLESS STEEL

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The present paper is focused on elaboration of phosphate masses with good molar ratios on SS 316L, with fluoride, $Zn^{2+}$ and $Cu^{2+}$ as dopant ions, using an electrochemical procedure and also the attempt to select the best conditions for stainless steel**.** The surface was characterized using X-ray difraction, AFM and contact angle measurements. X-ray has evidenced crystaline phases, contact angle measurements has established the balance hydrophyl-hydrophob and AFM established roughness values. The inductively coupled spectrometry (ICP-MS) has quantified the amount of cooper and zinc ions released. In order to be used in biomedical applications hemolysis and antibacterial tests have been performed.

(Recieved February 10, 2014; Accepted April 17, 2014)

*Keywords:* Electrodeposition, SS 316L, FHA, X-ray difraction, Contact angle measurements

## 1. Introduction

Metal allergy due to the ions released from dental and orthopaedic implants and generally speaking for all medical devices is dangerous for human health [1]. The trend of alloying elements in the last decades took into consideration this aspect, leading to the development of more stable alloys with non toxic and non allergenic elements, both for titanium alloys [1,2 ] and for cheaper alloys as stainless steel [3]. Due to the fact that the population ratio of the aged people with less income and more need of implant is increasing, the extension in use as implant materials of stainless steel and CoCrMo alloys is increasing as well [4,5,6,7] Their investigation has been extended due to the various possibilities to enhance surface properties of such cheaper implant bioalloys comparing with other metallic materials of the century, meaning titanium and its alloys. Having good corrosion resistance in bioliquids and mechanical properties that match with the human bone, such biomaterials represent a choise with a good ratio quality/ price and are more and more used in dental and othopaedic applications [3,6]. The mixture of native passive oxides layer formed at their surface is responsible for metallic alloys stability [7,8] and a large variety of surface modifications has been applied in order to improve their performance regarding wear and corrosion at micro and nanolevel and biocompatibility [ 9-11]. A great number of modifications was based on bioactivation with components of bone as collagen and hydroxyapatite [12-14] or other doped phosphate masses [15-17] taking into account that such coatings can increase the biocompatibility or antibacterial effect as well. Relatively new procedures consist on the introducing to the HA crystal structure chemical elements such as Fluoride, Silver, Zn.etc. [18], able to substitute $OH^-$ groups with $F^-$ ions and $Ca^{2+}$ with $Zn^{2+}$, $Cu^{2+}$ or Ag[18].

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Considering the merits and demerits of silver nanoparticles which can increase antibacterial effect [19,20] decreasing biocompatibility, a combined coating HA and Ag nanoparticles seems to be a good choise. Copper can be a promising metal ion for deposition applications because of its low eukaryotic cell toxicity and high cytocompatibility [21], and also can be easely metabolized [22] whereas silver tends to stay in the human body and increases the silver serum levels [23]. Application of fluoridated hydroxyapatite (FHA), $Ca_{10}(PO4)_6(OH)_{2-x}F_x$ where $0 < x < 2$ is the degree of fluoridation (FHA) as bioactive coatings in comparison with HA coating, could provide lower dissolution and better apatite-like layer deposition, and usually a better cell response [15-17]. The procedure was established for titanium substrate, but in this idea the present paper is focused on elaboration of phosphate masses with good molar ratios on SS 316L, with fluoride, $Zn^{2+}$ and $Cu^{2+}$ as dopant ions, using an electrochemical procedure and also the attempt to select the best conditions for stainless steel**.** The regression equation was established for electrodeposition and the surface was characterized using X-ray difraction and contact angle measurements. X ray has evidenced crystaline phases and contact angle measurements has established the balance hydrophyl-hydrophob. The inductively coupled spectrometry (ICP-MS ) has quantified the amount ofcooper and zinc ions released. In order to be used in biomedical applications antibacterial and hemolysis tests have been performed.

## 2. Experimental

### Sample preparation

Stainless steel 316L rectangular plates were polished with SiC emery paper from 800 down to 3000 in order to obtain a mirror like surface. They were washed in distilled water, degreased in acetone for 10 minutes, ultrasonically cleaned in ethanole to remove particles from the SiC abrasive papers, rinsed with ultrapure water and dried in air at room temperature.
We prepared the electrolyte using the composition found in table 1. In this solution were added 50 ppm of $ZnCl_2$ and/or $CuCl_2 \cdot 2H_2O$ in order to obtain FHA dopped with $Zn^{2+}$ or $Cu^{2+}$ and both $Zn^{2+}$ and $Cu^{2+}$.

*Table 1 The content of 500 ml electrolyte used for electrodeposition*

| **No.** | Substance | Concentration (mol/L) | Quantity (g) |
|---|---|---|---|
| 1 | $(NH_4)_2HPO_4$ | 0.025 | 1.6482 |
| 2 | $CaCl_2 \cdot 2H_2O$ | 0.042 | 3.0874 |
| 3 | $NH_4F$ | 0.012 | 0.2222 |

The pH of electrolyte solution was 4.8. In order to enhance the deposition process of dense coating by the release of $H_2$ gas and production of $HO^-$, $H_2O_2$ 3% was added (1 $H_2O_2$ :10 Electrolyte). $H_2O_2 + 2e^- \rightarrow 2HO^-$

Chemical composition of working electrodes is 0.08%N; 2.2% Mo, 0.01%S, 0.02%P, 0.067Si, 1.61% Mn, 11.81%Ni, 16.18%Cr, 0.027%C, Fe balance.

### Coating process

The coating process was performed at 30°C, under continous stiring using an electrochemical cell with platinum plate as counter electrode (anode), Ag/AgCl as reference electrode and SS316L as working electrode (cathode). The experiments were performed using a VoltaLab PGZ 301 potentiostat/ galvanostat controlled by a computer equipped with VoltaMaster 4 Software. In table 2 are presented the working parameters. A Medcalc program was used for description of coating process in time.

*Table 2. Working parameters*

| Sample | Current density [mA/cm$^2$] | Electrodeposition time [min] | Temperature [$^0$C] | Termic Treatment/ Time [$^0$C]/ [min] |
|---|---|---|---|---|
| SS316L | -1 | 20 | 30° | 130/ 360 |

After electrodeposition all samples were thermal treated in order to increase the crystallinity and the purity. Calcination was beneficial for strengthening the bonds of apatite coatings.

**Coating characterization**

The diffraction phenomenon occurs as a result of X-ray interaction with the electrons of atoms. Examination by X-ray diffraction is non-destructive and is applicable to a broad category of materials: bulk or consolidated, organic or inorganic, crystalline or amorphous and also nanomaterials. This technique allows to obtain a variety of information: identification and quantification of crystalline phases, assessing the percentage of amorphous material relative to the total quantity of crystalline material, the determination of network parameters, the determination of the crystal structure, etc.

The coating caracterisation was performed using an D8 DISCOVER ( Bruker Axs ) Difractometer,using as X-ray source a cathode from Cu(Kα=1.54060Å), mirror Gőbell - 0.6 mm and Lynx-Eye detector 1D, according to SR EN 13925:2003: Non-destructive testing. X-ray diffraction from polycrystalline and amorphous materials.

The diffraction condition is given by the law of Bragg:

$\sin\theta = n\lambda\ 2d_{(hkl)}$; λ - wavelength of radiation; θ - the angle of diffraction ; n - order diffraction with values 0, 1, 2, ... d - distance between planes (hkl)

Data acquisition was made in configuration Bragg – Brentano θ-2θ, in a $2\theta=10^0$-$70^0$ scanning range , measuring time 5 sec/step, angular increment 0.04°.

The incorporation of fluoride into the HA matrix did increase the crystallinity of the host crystals, and this fact correspond to the increased thermal and chemical stability of the FHA ceramics[24]

The phases were identified in comparison with Powder Diffraction File database from International Centre for Diffraction Data ( ICDD).

**Contact angle and roughness measurement.**

In order to evaluate the hydrophilic-hidrophobic balance wettability measurements on the modified surface have been performed determining the contact angle (CA) values with an 100 Optical Contact Angle Meter – CAM 100. Each contact angle determination value is the average of minimum three measurements. The tests were carried out with an accuracy of ±1° at 25 °C. The three-dimensional surface roughness (Ra) of the samples was evaluated by an atomic force microscope from A.P.E. Research Company. The atomic force measurement were performed in contact mode.

**ICP-MS measurements**

ICP-MS technique was used both for the determination of Zn and Cu content, and for determining the rate of detachment of these ions from the coating deposited on the SS 316L substrate.

Ion release was determined with ICP-MS, a sensitive method to detect a wide range of elements with a concentration down to nanograms per liter (ppt; parts per trillion) and below. The ICP-MS- equipment was a Perkin Elmer ELAN DRC-e instrument used with liquid sample introduction by a micro-nebulizer. Calibration was against aqueous multi-element solutions for internal standardization. All results are blank-subtracted averages of three replicate measurements. Analytical quality control was monitored by multiple analyses of procedural blanks, unknown samples, and certified reference materials NIST (National Institute of Standards and Technology

)1643e, IAEA W4. The samples of coated SS 316L were soaked into 10 ml of simulated body fluid (SBF) in polystyrene sterile flasks for 1, 4, 6, 12, 24, 48, 72 hours at 37 ◦C. SBF solutions were prepared according to Kokubo's SBF solution [25]

In order to determine the content of Zn and / or Cu in the coatings SS316L samples, they were treated with nitric acid to dissolve the deposited layer.

**Haemolysis tests**

The destruction of the membranes of red blood cells and the release of haemoglobin in the surrounding fluid considered as a measure of the biomaterial cytotoxicity over the red blood cells was performed according to the Drabkin method [26]. The quantity of hemoglobin liberated in the plasma reported to the total amount of haemoglobin from the blood sample was determined.

The materials were evaluated to determine if they are compatible with blood or they can produce hemolysis in vitro, by extraction or direct contact.The hemolytic activity of the samples was investigated according to to ASTM F 756-00[27] and ISO 10993 – 4[28].

According to ASTM F 756-00 all materials can be classified in three different categories: haemolytic over 5% hemolytic index , slightly haemolytic between 5% and 2% , and non-haemolytic below 2%.

Blood sampling procedures were respected according to SR EN ISO 15189:2007 and SR EN ISO 15190:2005. The blood was collected in a clinical laboratory by specialized personnel from a healthy woman, 38 y.o.,B III blood type, Rh positive, HIV 1,2 -negative, Syphilis negative, anti-HCV, HBsAg and HAV-IgM-negative, non-smoker, non-alcoholic , no drug intake in the last 10 days before blood sampling. The blood was drawn by venipuncture in Beckton-Dickinson vacuum tubes containing sodium citrate (3.8%) in a proportion of 9:1.

Positive and negative controls were prepared by adding 1 ml of blood in 7 ml SWFI (Sterile Water for Injections), produced by Zentiva, and the same volume of blood in 7 ml CMF-PBS.

The samples for direct contact were imersed in 10 ml of „in house" prepared CMF-PBS(calcium and magnesium free -Phosphate Buffered Saline) for 72h at 37°C.They were put in contact with 1 ml fesh citrated blood.The samples for extraction were covered with 10 ml CMF-PBS and incubated at 121° for 1 h, slightly agitatig from time to time. An aliquot of 7ml was taken from each recipient and 1 ml of blood was added. All samples and controls were incubated 3 hours at 37°, sligtly agitating every 30 minutes. After incubation, they were centrifuged at 3500rpm for 15 minutes, and the supernatant was separated. The hemolytic activity was determined using the Drabkin method , the optic density being read at 546 nm with an automatic analyzer Prestige 24i. The formula used for calculation was:

$$\text{Haemolysis}(\%) = \frac{(\text{OD}_{\text{sample}} - \text{OD}_{\text{negativ control}})}{(\text{OD}_{\text{pozitive control}} - \text{OD}_{\text{negativ control}})} \times 100$$

**Antimicrobial activity**

Heavy metals are toxic and react with proteins; therefore they bind protein molecules; as a result cellular metabolism is inhibited causing death of microorganism [29].

ZnO nanoparticles have bactericidal effects on both Gram-positive and Gram-negative bacteria. They even have antibacterial activity against spores that are resistant to high temperature and high pressure [30]. The adhesion of ZnO particles on the membrane of bacteria could be the underlying cause of zinc toxicity effect towards the bacteria [31].

The implant materials should aim at the implementation moment of an antibacterial effect while displaying a high degree of cytocompatibility [32].

We used two of the most encountered Gram-negative and Gram-positive bacteria in implant related infections using reference strains from American Type Culture Collection. Meticillin-resistant Staphylococcus aureus (MRSA) is resistant to all ß-lactam antibiotics, but some results demonstrateed an antimicrobial effect of copper on MRSA [33], P.aeruginosa infections are often difficult to treat, and numerous therapeutic options, besides antibiotics, have been searched, even quorum-sensing inhibitors [34] *Staphyloccoccus aureus* (ATCC 25923) and *Pseudomonas aeruginosa* (ATCC 27853) were aerobically cultured for 24 h at 37 °C on Columbia sheep blood agar plates. From the pure fresh cultures 3-4 colonies were selected and

inoculated in standard saline solution from Fresenius Kabi, the concentration was adjusted to 0.5 McFarland units, meaning aproximatly $1.5x10^{-8}$ CFU (colonies forming units). For each bacterial strain new samples were placed in sterilized glass tubes of 18mm diameter, and 10 ml of infected medium was pourred in, the positive control tube contained only infected medium, the blank tube contained only saline solution . The tubes were incubated 24 hours at 37°, slightly agitating from time to time.

The optical densities were read at 600nm against blank- sterile saline solution with an UV–VIS Jenway Spectrophotometer.

The antibacterial activity was determined by calculating the bacterial growth inhibition index using the formula:

$$I\% = \frac{(C_{24} - C_0) - (S_{24} - S_0)}{(C_{24} - C_0)} \times 100$$

where I is the growth inhibition activity, $C_{24}$ and $C_0$ are the blank-corrected optical densities of the control at time 0 and after 24 h, $S_{24}$ and $S_0$ are the blank –corrected optical densities of infected media in the presence of test samples at initial time and respectively at 24 h.

## 3. Results

Figure 1 shows electrochemical deposition of fluoride apatite coatings on SS316L samples.

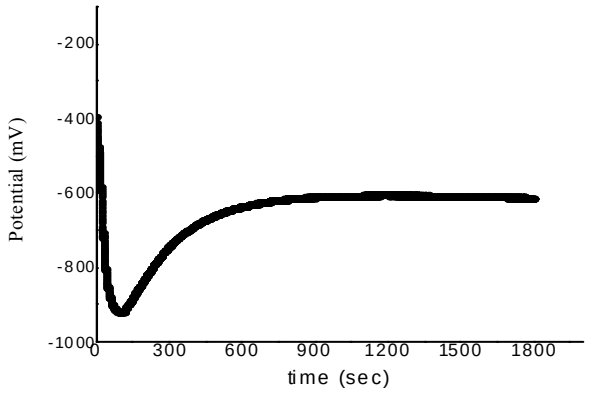


*Fig1. The dependence potential versus time in the coating process SS 316L*

The equation obtained with a Medcalc program is presented in table 4 together with R, the coefficient.

*Table 4 Curve equation*

| Sample curve | Equation | $R^2$ |
|---|---|---|
| SS 316 L | $y = -0.80368 + 0.1388 \cdot e^{-0.07442x}$ | 0.85 |

**XRD-results**

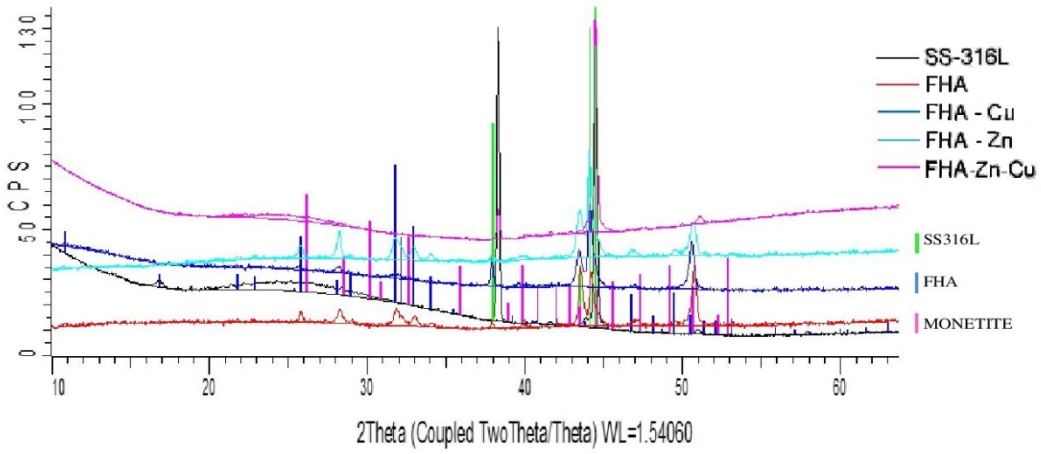


*Fig 2.The compared diffractograme of FHA, and Cu and/or Zn dopped FHA*

From the obtained diffractograme we can conclude that the obtanined coating is composed only from fluorohydroxyapatite (FHA), except for the samples covered with florohydroxyapatite with Cu (FHA-Cu) where we have also monetite wich is considered the precursor for hydroxyapatite (HA) [35] maybe this is due to an incomplete transformation during calcination, for evaluate this situation further studies will be made. HA has hexagonal cristalization system (a=b≠c, α=β=δ=90°, γ(x,y)=120°). The obtained peaks for FHA were at 2θ = 25,760$^o$ ,28,115$^o$ , 31,817°,32,938$^o$, 46,679$^o$, the main peak position of the fluorohydroxyapatite is at 2θ =31,817°, and the interplanary distance d= 2,81031 Å, after which the structure is guided and is associated with the crystallographic plane (h, k, l) - (2,1,1), where h, k, l are Miller indices associated to the crystallographic plane.

The FHA layer is different from HA, the angle has moved to a higher value compared with the spectra of hydroxyapatite given by the ASTM card (from 31.766° to 31.817°) .

The obtained peaks for monetite were at 2θ=28,452$^o$, 30,134$^o$, 30,863$^o$, 32,602$^o$ and 39,836$^o$ .

**Contact angles and roughness measurement**

Contact angle is an essential method to assess the surface treatment efficacy [36], and surface roughness is one of the most important topographical parameter influencing cell–material interactions. In Table 4 are presented the values of contact angle and the roughness for different types of coatings on stainless steel substrate.

*Table 4. The values of contact angles and the roughness*

| Coating | CA | $R_a$ (μm) |
|---|---|---|
| 316L/FHA | 25 | 0.402±0.35 |
| 316L/FHA-Cu | 34 | 0.385±0.28 |
| 316L/FHA-Zn | 34 | 0.391±0.4 |
| 316L/FHA-Cu-Zn | 38 | 0.380±0.22 |

The surface roughness measurements did not show statistically significant differences between the roughness values of samples coated with doped fluorohydroxyapatite. The decreased contact angle reveals the increased surface roughness.

## ICP-MS MEASUREMENTS

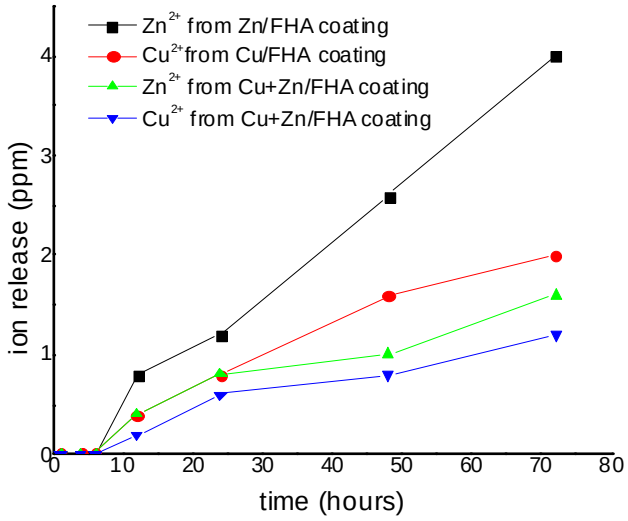


*Fig.3. Evolution in time of ions release process*

After ICP-MS determinations, the content of Cu and Zn ions detected in 316L stainless steel coatings were: 24 ppm Zn in FHA- Zn, 14 ppm Cu in Cu FHA -Cu coverage, 10ppm Zn and 8 ppm Cu in FHA –Cu-Zn/

## HEMOLYSYS

According to Standard Practices for Assessment of Haemolytic Properties of Materials; materials can be classified in haemolytic with a percentages of haemolysis over 5% , slightly haemolytic with a haemolytic index between 5% and 2% , and non-haemolytic with the haemolysis percentage below 2%. The haemolytic tests indicate that direct contact and extraction for FHA, FHA+Cu and FHA+Zn+Cu are non hemolytic, but in case of FHA+Zn the extract is not hemolytic but the direct contact proved to be slightly hemolytic.The obtained rezults are shown in Table 6.

*Table 6. Blank corrected hemolityc index*

| Sample | FHA | FHA-Cu | FHA-Zn | FHA-Cu-Zn |
|---|---|---|---|---|
| HI% Direct | 0.725 | 1.209 | 2.131 | 1.639 |
| HI %Extract | 0.053 | 0.106 | 0.214 | 0.106 |

## Antibacterial effect:

The inhibition level, (% ) is presented in table 5

*Table 5.The bacterial growth inhibition ratio*

| Sample | FHA | FHA-Cu | FHA-Zn | FHA-Cu-Zn |
|---|---|---|---|---|
| I% S.aureus | 7.27 ±0.16 | 63.626 ±1.41 | 61.818±2.08 | 62.72±2.11 |
| I% P.aerug | 10.9 ±0.19 | 54.545 ±1.15 | 43.636±1.17 | 52.72±1.58 |

The presented results are media of five replicates. The non dopped FHA has a slightly antibacterial effect , due probably to the fluorine ions.In case of Cu dopped FHA , both in gram positive and gram negative bacteria the inhibition index is quite high. In Zn dopped FHA , in P.aeruginosa case , compared to S. aureus, the ihibition is the lowest observed, due probably to the difference in the protein constituents of their cell walls [37].

## 4.Discussion

According to the Fig 1 at the beginning of the electrodeposition process the potential values are decreasing rapidly to more electronegative values until a plateau is reaching when for more than 3500 seconds tends to remain at the same values. There is a tendency to a steady state representing the coating formation which is characteristic for electrodeposition on SS 316L. The equation representing the process evolution is an exponential type with an R coefficient 0, 85.

The very high hydrophilic character of the coating according to the contact angles values should be as well a support for confirming osteogenesis. Knowing that cell do prefer such surfaces, the electrodeposited phosphate masses is going to promote osteoblast cell growth.

Such properties are sustained with other aspects of the mineralization properties related to fluoride presence. The deposition is taking place according to the reaction :

$$Ca_{10}(PO_4)_6(OH)_2+2xF^-+2xH^+ \rightarrow Ca_{10}(PO_4)_6(OH)_{2-x}F_x+2xH_2O.$$

Knowing from the literature (20) and from our that coating elaboration of FHA is taking place under cathodic control the electron transfer at the interface substrate electrolyte is promoting $H_2PO_4^-$ dissolution and FHA crystals are growing directly on the substrate simultaneously with over saturation according ro the global equation:

$$10Ca^{2+}+6PO_4^{3-}+xF^-+(2-x)\,OH^- \rightarrow Ca_{10}(PO_4)_6(OH,F)_2$$

**ICP-MS measurements**

The high concentration of $Zn^{2+}$ in both Zn–FHA and Cu/Zn–FHA coatings, this may be explained by the ease substitution of $Zn^{2}+$ with $Ca^{2+}$ compared with $Cu^{2+}$ which due to the difference of the properties of these ions. Mhammedi et al. [38] have showed that the substitution of $Ca^{2+}$ by $Cu^{2+}$ in the synthetic hydroxyapatite is very limited.

Regarding metal ion detachment from the coatings, Fig.3 shows the time evolution of this process.

Ions release test showed that doped FHAP samples in SBF released no detectable amounts of copper or zinc ions after 1, 4 and 6 h. Futures studies are needed to detect the exact optimum of Zn and Cu concentration in FHA coating is needed to achieve the best biological response.

**Hemolysis tests**

The hemolysis tests show that all extraction solutions have no hemolytic effect, and the same result was obtained in case of direct contact of the blood with FHA, FHA-Cu and FHA-Cu-Zn. As it can be seen in table 6 the sample covered with FHA-Zn has a very slightly hemolytic effect. Besides other indexes of cytotoxicity, such as release of LDH (lactate-dehydrogenase), the hemolysis can be considered a measure of the cytotoxicity over the red blood cells. Other authors observed that the presence of $Zn^{2+}$ increases, in a dose-dependent way, the release of LDH in the extracellular medium [39], an oxidative damage triggered by zinc has been observed in endothelial cells [40], retinal cells [41] and peripheral blood lymphocytes [42].

**Antibacterial effect**

Biofilm forming bacteria cause persistent infections that are difficult to treat using the classical antibiotics administration, because the extracellular matrix acts as a physical barrier preventing the penetration of antibiotics into the biofilm [43], so another approach is needed, for example an implant anti-infectious surface coating, in order to prevent microbial attachments and proliferation and therefore biofilm formation, in order to prevent early and delayed postoperative implant-associated infections. The antibacterial effect of copper coated surfaces and its influence on S. aureus biofilm formation is well established [44], but higher concentrations of copper are

toxic for any type of cells , DNA damage representing a major component of copper toxicity [45]. In case of P. aeruginosa, depending of the method of inoculation-spreading or dropplet, on copper coupons the bacteria survived only 2 to 4 hours, after 4h the culture being completely inactivated [46].

In dentistry, the antibacterial effects of zinc oxide have been reported from long time ago [47], however according to several authors, the presence of Zn cathions seems to negatively potentiate the activity of imipenem against P.aeruginosa [48],and also aminoglycosides activity [49].

## 5. Conclusions

The phosphates masses doped with fluoride, copper and zinc ions on stainless steel were elaborated via electrodeposition and calcination.The coatings have hydrophylic character and the investigation using X-Rays has indicated FHA for all doped phosphate masses excepting the FHA-Cu where a precursor of HA, monetite was observed. Regarding ICP-MS tests, the doped FHA samples in SBF released no detectable amounts of copper or zinc ions after 1, 4 and 6 h.The non dopped FHA has a slightly antibacterial effect, due probably to the fluoride ions, but in case of Cu dopped FHA, the inhibition index is quite high. With Zn ions dopped FHA, the inhibition is lower. This coating is the only one with hemolytic index higher than 2, as an expression of a slight hemolytic character.

### Acknowledgments

Georgeta Totea wishes to thank The European Social Fund, Sectoral Operational Programme: Human Resources Development, 2007–2013, of the Romanian Ministry of Labour, Family and Social Protection, through the Financial Agreement POSDRU/107/1.5/S/76813 program. All authors wish to thank to Romanian- Argentina bilateral program as well.

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