BABEŞ-BOLYAIUNIVERSITY FROM CLUJ-NAPOCA
FACULTY OF CHEMISTRY AND CHEMICAL ENGINEERING
DEPARTMENT OF CHEMICAL ENGINEERING
SCIENTIFIC RESEARCH CENTER IN PHYSICAL CHEMISTRY

ABSTRACT OF DOCTORAL THESIS

PREPARATION AND CHARACTERIZATION OF NANOHYDROXYAPATITES DOPED WITH VARIOUS BIOLOGICAL ACTIVE COMPOUNDS WITH BIOMEDICAL APPLICATIONS

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CONTENTS

Keywords
stoichiometric hydroxyapatite; multisubstituted hydroxyapatite, magnesium, zinc, silicon, strontium; silver nanoparticles; vancomycin; collagen; PLA; XRD; SEM-EDX; AFM; Ti implants; Ti implants coated with biomimetic composites; biocompatibility; antimicrobial activity; *in vivo* rat model; femoral fracture; ALP; OCN; histological analysis; micro-CT; mechanical properties; new bone formation; bone fracture healing.

Introduction

Bone replacement and bone repair are the most controversial methods of bone regeneration in orthopedic surgery. For medical applications, it is necessary to develop materials that mimic chemical composition and structure of natural bone and favour faster healing with minimal side effects. The bone of the human body is one of the largest organs that perform the functions of movement, support, mineral intake and protection. It also has the ability to reshape and self- healing that means absorption of old or of damaged bone tissue occurring at the same interface where osteoblasts produce new healthy bone to replace resorbed bone. However, in the critical cases when the bone is severely damaged, self-healing is not enough. Therefore, biomaterials capable of substituting or regenerating the affected bone tissue need to be developed.

Basically, the bone tissue engineering seems to solve these problems utilizing a combination of multidisciplinary approaches to improve or replace damaged bone tissue. In recent years, due to the development of tissue engineering technology, bone tissue engineering has become an optimistic approach for repairing bone fractures and bone defects. Biomaterials for bone substitutes and for coating of metallic implants or as scaffolds in cell culture were developed in this thesis [1-14] and play a crucial role in bone tissue engineering. Their purpose is to mimic the composition, structure and function of the natural bone and provide a three-dimensional (3D) environment to have adequate physical properties for bone repair promoting the adhesion, proliferation, and differentiation of osteoblast cells *in vivo*[5,6].

An ideal nanostructured biomaterial, also named biomedical material should be biodegradable, bioactive, biocompatible and osteoconductive or especially osteoinductive *in vivo*, proving the new bone formation and growth. The goal of this work was to design, prepare and characterize synthetic nanohydroxiapatites, nano HAPs, which are important components of natural bone [1-14], doped with various biological active compounds, such as collagen (COL, another essential component in bone), and drugs (e.g. antibiotics, like vancomycin and silver nanoparticles:

AgNPs to protect against infections [6, 8] jointly able to develop innovative nanomaterials with enhanced biological functions for bone substitutes and for coating of metallic implants with potential biomedical applications, for biological bone repair, enhanced bone fracture healing and bone regeneration [7, 9, 12, 14].

Due to the various needs of synthetic nanostructured biomaterials, like nanohydroxiapatites multi-doped with essential physiological elements, such as Mg, Zn and Si, resulting multi-substituted hydroxyapatites, noted ms-HAP or HAPc, were also developed in this work to enhance the bone regeneration [1-14].

The multidisciplinary approaches developed in this work can be applied for bone regeneration and will make important steps in the near future concerning the exploitation of novel biomaterials and new strategies regarding the integration of nanotechnology, in stem cell science for bone tissue regeneration helping the population at risk, such as patients with osteoporosis.

The design, synthesis, and physico-chemical characterization, as well as their biological behavior were carefully investigated in this doctoral thesis under the leadership of Univ. Prof. Dr. Maria Tomoaia-Cotisel, Director of the Research Center in Physical Chemistry, CECHIF, at Babeş-Bolyai University of Cluj-Napoca. The results obtained in this doctoral thesis are part of significant achievements obtained in the last decade in the CECHIF center, including *in vivo*studies using *femoral fracture rat model*.

Chapter 1 [7] aims to investigate *nanostructured biomaterials* made within hedoctoral research, *nanostructured biomimetic composite* materials, containing three components: HAPc nanoparticles functionalized with COL crown (core / shell nanoparticles), incorporated in polylactic acid, PLA, resulting in porous coatings HAPc-COL @ PLA on Ti implants. Finally, these composites were coated with self-assembled COL fibers, resulting in HAPc-COL@PLA/COL biomimetic materials [7]. The addition of collagen to the porous HAPc-COL@PLA coating material increased the mechanical strength of the composite, causing a reduction in its porosity. The materials were obtained by the self-assembling dip coating method on Ti implants. The materials were characterized by top methods and techniques, namely XRD, TEM, SEM, EDX, AFM (Figure 1), and methods for determining the mechanical properties (Figure 5, Table 1) of the implants used. The materials were studied *in vivo*, on a *model of femoral fractures in rats*which represents a *premiere in the international scientific community*.

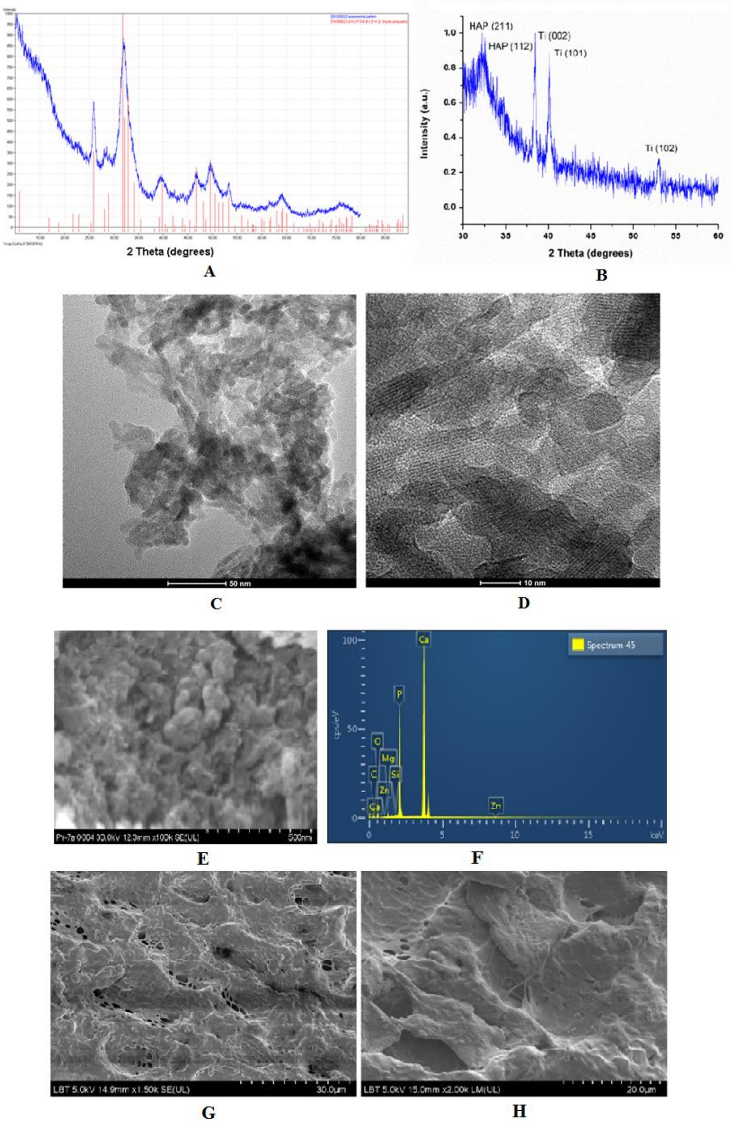

Figure 2. XRD patterns for the lyophilized ms-HAP/COL (A) powder and for the HAPc composite (i.e., the ms-HAP/COL@PLA/COL coating) on the Ti surface(B); HR-TEM images (C, D) : shapes of HAP/COL nanoparticle; SEM image (E) and EDX spectrum for the same area (F) for HAP/COL; SEM images (G, H) of the HAPc coating on Ti surface.

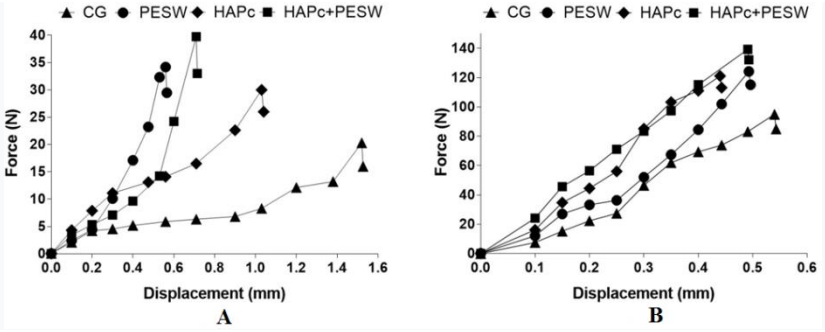

Figure 5 Force vs displacement curves for the three points bending tests performed on the explanted femur of rats: (A) at 2 weeks post-implantation and (B) at 8 weeks post-implantation

Table 1 Measured values of the breaking force and the corresponding displacement during the three points bending tests.

Group		CG	PESW	HAPc	HAPc+PESW
Ultimate force[N]	2 weeks	20 ± 5	34 ± 4	30 ± 4	40 ± 5
	8 weeks	95 ± 9	124 ± 6	121 ± 8	139 ± 8
Ultimate displacement [mm]	2 weeks	1.5 ± 0.3	0.6± 0.2	1.0 ± 0.3	0.7 ± 0.1
	8 weeks	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.2	0.5± 0.1

Abbreviations: CG = control group; PESW = pulsed electromagnetic short-waves; HAPc = titanium implants coated with multisubstituted hydroxyapatite and collagen; HAPc+PESW = titanium implants coated with multisubstituted hydroxyapatite and collagen and pulsed electromagnetic short-waves; N = newton; mm = milimeter.

The results regarding an *in vivo* evaluation of the enhancement of bone consolidation using highfrequencypulsed electromagnetic short-waves and titanium implants coated with biomimetic composite embedded into PLA matrix are presented. This research demonstrates the importance of biomimetic coatings deposited on titanium implants in the rapid healing of femoral fractures in the rat model. The multidisciplinary research carried out includes the determination of bone markers: alkaline phosphatase and osteocalcin, at different implantation times in animals (at 2, 4 and 8 weeks, respectively); the technique of micro computer-tomography (micro-CT and the histological analysis) are used, and the fast healing of the femoral fractures in the rat is highlighted.

Chapter 2 [9] reports on *Biocompatibility of titanium implants coated with biocomposite in a rat model of femoral fracture*and demonstrates *in vivo*the osseointegration of the biomimetic coating on Ti implants with the host natural bone. Measurements of bone marker biophysics: alkaline phosphatase and osteocalcin (Figure 2, Table 1), as well as micro-CT (Table 2) and histological analysis (Figure 3). They demonstrateosseointegration (Table 2), the formation of trabecular bone and compact bone - facilitated by the biominetic structures designed and developed in this doctoral thesis.

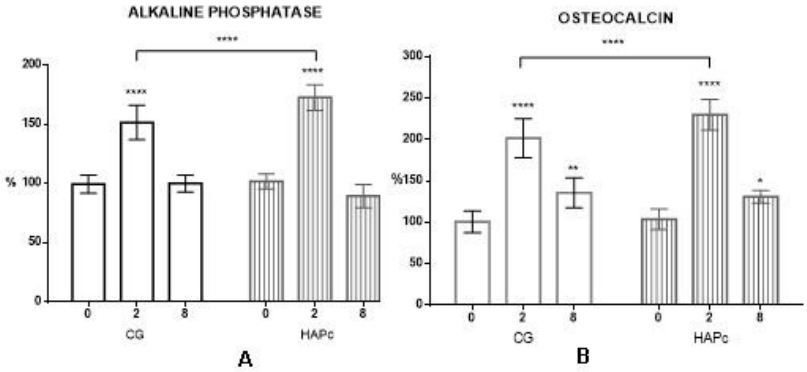

Figure 2. Bone markers, alkaline phosphatase (A) and osteocalcin (B), serum concentration at zero time (initially), two- and eight-weeks post-surgery; *statistically significant p<0.05; **statistically significant p<0.01; ***statistically significant p<0.001; ****statistically significant p<0.0001

Table 1. Bone markers, alkaline phosphatase (ALP) and osteocalcin (OCN), serum concentration at initial (0 weeks), two- and eight-weeks post-operatively; *statistically significant p<0.05; **statistically significant p<0.01; ***statistically significant p<0.001; ****statistically significant p<0.0001

Rat group		CG	HAPc
ALP (%)	0 weeks	100 ± 13	102 ±6
	2 weeks	152 ±14****	173 ± 10****
	8 weeks	100 ± 7	89 ± 8
OCN (%)	0 weeks	100 ± 15	104 ± 13
	2 weeks	202 ± 24****	230 ± 18****
	8 weeks	136 ± 18**	131 ± 8*

Table 2. Implant osseointegration assessed by micro-CT; bone volume per total tissue volume (BV/TV) and the mean trabecular number (Tb.N); $^{*}p < 0.05$: HAPc group vs CG.

Rat group	CG	HAPc
BV/TV(%)	25.5±4.3	38.8±5.4*
Tb.N (1/mm)	154±18	180±18*

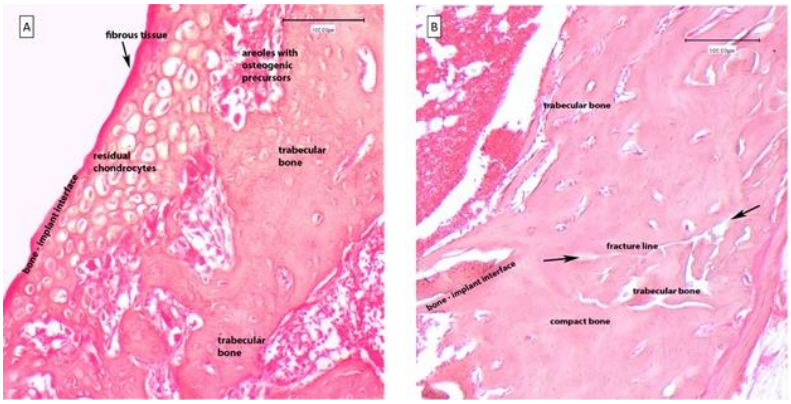

Figure 3. Optical microscopy images of H&E stained slides of tissue samples at bone-implant interface near the fracture site, at eight weeks after implantation. (A): control group revealed fibrous tissue in proximity of Ti intramedullary implant and residual cartilaginous tissue indicating a transition from cartilaginous precursors to incipient bone trabeculae formation. (B): HAPc group displayed around HAPcTi implants the bone trabeculae well defined, compact bone with lamellar disposition of bone matrix and osteocytes around Havers canals, with osteoblasts lining their surface, and clear delimitation of areole between the trabeculae with areas of compact lamellar bone deposition.

Chapter 3 continues with the exploration of the *Behavior of multisubstituted hydroxyapatites inwater and simulated body fluid* (SBF). In order to determine the ion release profiles of these substituted hydroxyapatites [3]. Substituted nano-HAPs containg Mg, Zn, Sr and Si are synthesizes and characterized and it was demonstrated that substitution with essential elements in the HAP structure leads to the controlled release of the constituent ions from the HAP lattice, depending on the amount of doping element. While in water the release of the constituent ions is observed (Figure 6), in SBF this process is countered by the uptake by the sold HAP of Ca, Mg and Si from the solution (Figure 7). The long time release observed for the valuable physiological elements contained in multisubstituted hydroxyapatites evidences a promising future of these biomaterials for biomedical purposes.

Magnesium is found in bones and teeth. It is involved in the growth and remodeling of bones by activating osteoblast cells. Magnesium deficiency is linked to the onset of osteroporosis. *Zinc* inhibits osteoclasts and intensifies the response of osteoblasts. Anti-inflammatory and antimicrobial effects of zinc-substituted HAPs have also been reported. *Strontium* is known for its action on bone regeneration by increasing osteoblast activity and decreasing bone resorption by acting on osteoclasts. Strontium ranelate is increasingly used in the treatment of osteoporosis. *Silicon* is also involved in the bioactivity of osteoblasts.

For the synthesis of hydroxyapatites a solution containing the cations and one containing the anions were prepared. The 0.25 M cation solution contained Ca^{2+} and, in addition, for complex HAPs:Mg^{2+}, Zn^{2+} and Sr^{2+}, according to the composition to be obtained. It was prepared by dissolving in ultrapure water the nitrates: $Ca(NO_3)_2 \cdot 4H_2O$, $Mg(NO_3)_2 \cdot 6H_2O$, $Zn(NO_3)_2 \cdot 6H_2O$ and $Sr(NO_3)_2$. The 0.15 M anions solution contained PO_4^{3-} and (for complex HAPs) SiO_4^{4-}. The solution was obtained from diammonium hydrogen phosphate, $(NH_4)2HPO_4$ and tetraethyl orthosilicate, TEOS, $Si(OC_2H_5)_4$(98%) in the appropriate ratio. The working pH was 11.5, fixed by adding a 25% ammonia solution. Equal volumes of the two solutions were mixed rapidly at room temperature, (22^{o}C), using a peristaltic pump and a 'Y' type impact reactor for the two liquid flows. The suspension obtained was matured in two stages: at 22°C for 24 hours and at 70°C for another 24 hours, with intermittent stirring. The final precipitate was filtered and washed repeatedly with ultrapure water at room temperature until nitrates were removed. The drying process was carried out by lyophilization followed by the calcination step at 300°C for one hour and the sample as disintegrated in a ball mill to obtain a fine powder.

The presence of the HAP network as the unique crystalline phase was established by XRD and FTIR spectroscopy. The chemical composition was confirmed by SEM-EDX. TEM, SEM and AFM imaging showed the morphology of these biomaterials. The release of elements into water and simulated body fluid (SBF) was monitored over time, from 1 to 90 days, using inductively coupled plasma optical emission spectrometry (ICP-OES).

The amounts of Ca, P, Si and Mg in solutions were measured after immersion of 0.15 g of each sample in 15 ml of ultrapure water, respectively in Kokubo's simulated body fluid (SBF) and incubation at 37°C in separate closed flaskss for each sample/day. After 1, 3, 7, 14, 21, 30, 60 and 90 days, the supernatant (after centrifugation) was filtered. For calibration, standard multi-element solutions were prepared by diluting Merck IV multi-element stock solutions to 1000 mg/L. The Zn content in the aqueous phase was below the detection limit for all samples.

During the release of ions into water (Figure 6), for substituted hydroxyapatites, the amount of Ca released is higher than from pure HAP and very similar for all complex hydroxyapatites. The P / Ca ratio in solution is higher than in solid samples, which indicates an incongruent dissolution process. The amount of Mg passed into the aqueous phase is disproportionately high compared to its content in solid samples. It is lower for HAPc-Sr samples, denoting a stabilization of complex HAP by the simultaneous presence of Mg and Sr. Sr release, Figure 6d, increases slowly over time. It is higher for a higher Sr content in the solid sample. The Si content in solutions, Figure 6e, is almost constant over time after the first day. It is the highest for HAPc and decreases with increasing Sr content.

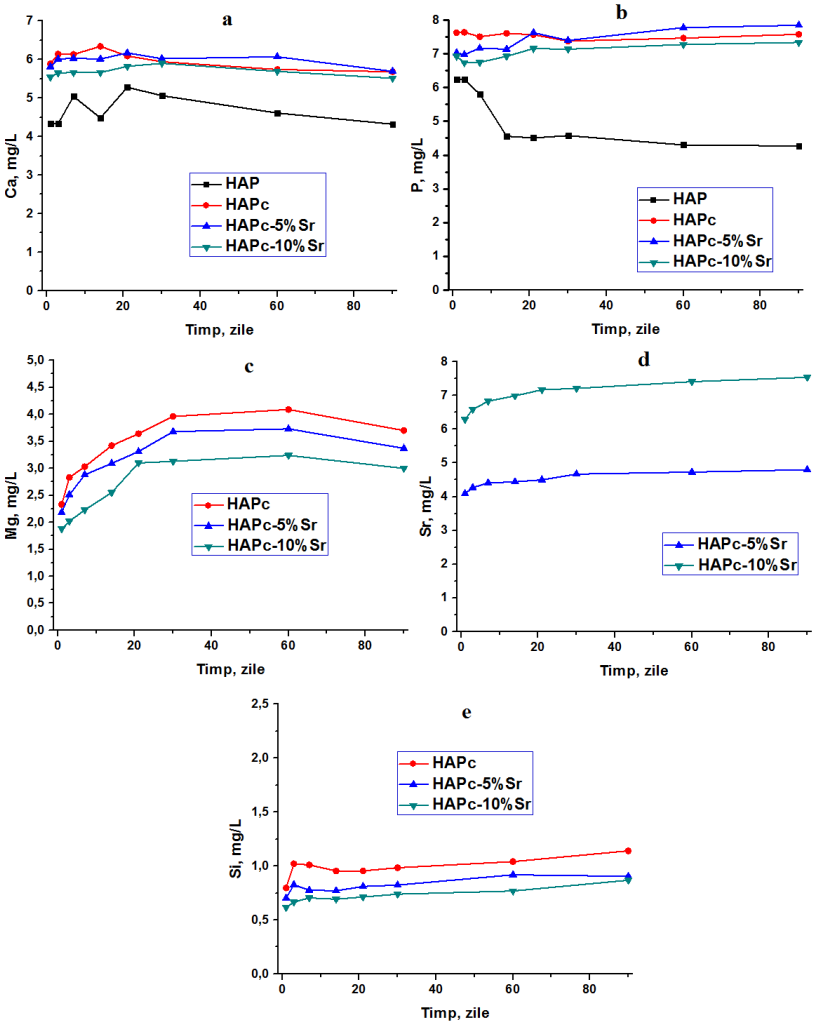

Figure 6. Calcium (a), phosphorus (b), magnesium (c), strontium (d), and silicon (e) release in water after immersion of HAPs samples for 1 – 90 days.

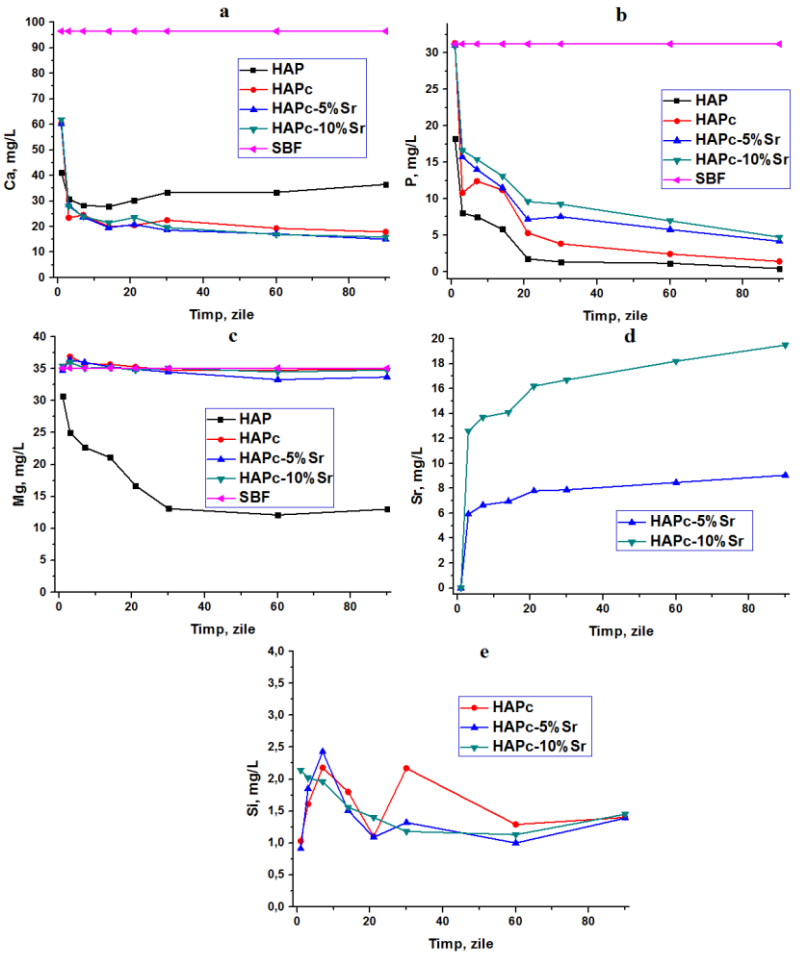

Figure 7. Calcium (a), phosphorus (b), magnesium (c), strontium (d), and silicon (e) contents in SBF after immersion of HAPs samples for 1 day to 90 days.

Chapter 4 presents the *Higuchi model applied to ions release from hydroxyapatites* [10] continues the investigation of ions release from tetra-substituted HAPs with Mg, Zn, Sr and Si in water and SBF, in static and simulated dynamic conditions. The results of the experimental ion release determinations were theoretically interpreted by a diffusion-based Higuchi kinetic model. By representing the amounts of ions released as a function of the square root of time,

the applicability of the Higuchi equation was verified (e.g. Figure 2 for simulated dynamic conditions).

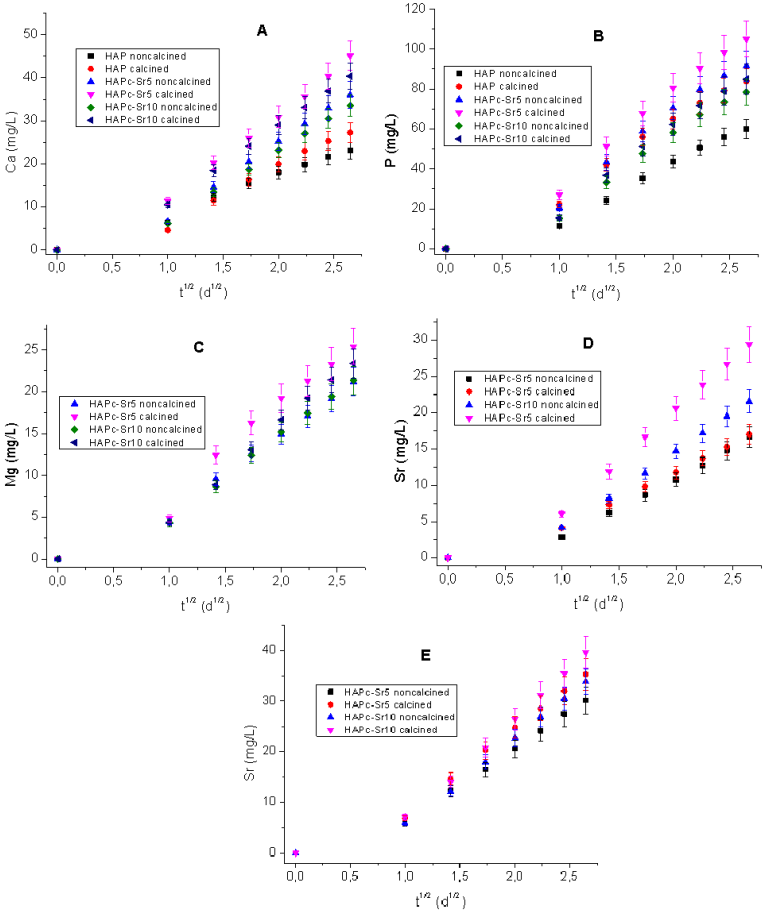

Figure 2. Cumulated ion release vs. time$^{1/2}$ (days$^{1/2}$) for 7 days in simulated dynamic conditions from noncalcined and calcined samples of HAP, HAPc-5%Sr (HAPc-Sr5) and HAPc-10%Sr (HAPc-Sr10). Release in water of Ca^{2+}(A), P (phosphate ions, B), Mg^{2+}(C), Sr^{2+}(D), and Sr^{2+} in SBF (E). Vertical bars represent the standard deviations of the measured values

The behavior of the synthesized stoichiometric hydroxyapatite (HAP): $Ca_{10}(PO_4)_6(OH)_2$ and two multisubstituted hydroxyapatites (ms-HAPs), both containing 1.5 wt% Mg, 0.2% Zn, 0.2% Si and different amounts of Sr: HAPc-5% Sr, respectively HAPc-10% Sr, when immersed in water and simulated body fluid

(SBF) has been recently researched [[1, 3, 4, 5, 10]. The theoretical formulas for ms-HAP materials are: $Ca_{8.76}Mg_{0.63}Zn_{0.03}Sr_{0.58}(PO_4)_{5.93}(SiO_4)_{0.07}(OH)_{1.93}$ for HAPc-5% Sr and $Ca_{8.12}Mg_{0.65}Zn_{0.03}Sr_{1.20}(PO_4)_{5.93}(SiO_4)_{0.07}(OH)_{1.93}$for HAPc-10% Sr. The release of Ca^{2+}, Mg^{2+}, Sr^{2+}, as well as P (phosphate) in water and the variation of ion content in SBF in contact with submerged HAPs samples was measured using inductively coupled plasma emission spectrometry (ICP-OES). Zn^{2+} and silicate ions could not be detected in the solutions, as they were below the ICP-OES detection limit. A static method was applied, in which the HAPs samples were kept in the immersion liquid in closed flasks for different periods of time, from 1 to 90 days, and a simulated dynamic method, when the immersion liquid was changed daily with a fresh one, for 7 days.

The Higuchi model is based on Fick diffusion, so it should be applicable when the internal diffusion of ions from the bulk of the particle to the surrounding liquid is the rate-determining step, and the released species is evenly distributed in a homogeneous matrix. In this case, the amount released of a species should be proportional to the square root of time. But the regression lines for the linearization $Mt = f(t^{1/2})$ do not pass through the origin of the coordinates, in agreement with the finding that diffusion is not the main process in the early stages of ion release. Consequently, a modified form of the Hguchi equation was tried

$$M_t = a + Kt^{1/2} \qquad (2)$$

where M_t is the cumulative amount of ions released at time t, and K is a rate constant of ion release, which depends on both the characteristics of HAP nanoparticles and on the properties of the released species, but also on the nature of the immersion medium and temperature.

Equation (2) is applied satisfactorily for the release of ions under static conditions, most of the values of the determination coefficient r^2 are over 0.9. The Higuchi equation applies better to Ca and P, the major constituents of hydroxyapatites, best to unsubstituted HAP. The equation fails in case of Sr release from HAPc-10% Sr in SBF, where ion exchange processes can occur between Ca^{2+} or Mg^{2+} ions in the solution and Sr^{2+} ions in the solid sample. Higher r^2 values are observed for the last days of interaction between hydroxyapatite and water in most ions and samples, the linearity of the representations $Mt = M_t = f(t^{1/2})$ becoming more evident, as can be seen in Figure 2.

Considering the parameter K as a measure of the diffusion rate, it results that the diffusion of calcium and phosphorus takes place faster from substituted HAPs than from the unsubstituted, while for HAPc-10% Sr the rate is lower than for HAPc-5% Sr. This could be explained by the distortion of the crystal lattice by substituting the Ca^{2+} ion with other cations of different sizes, which favors internal diffusion. On the other hand, the values of a (y-intercepts) can be considered as an extrapolation of the amount of ions released at time 0 and a measure of the initial solubility. For Ca^{2+} they are also higher in substitut HAPs, which confirms

the increase in solubility by substitution in hydroxyapatite. The diffusion rate of the Mg^{2+} ion is much higher than that of Sr^{2+} and also above the value for Ca^{2+}. This may be due to the smaller size of the Mg^{2+} ions (ionic radius 86 pm) compared to Sr^{2+} (132 pm) and Ca^{2+} (114 pm), and therefore the higher mobility of the former. Higher strontium release in SBF than in water can be attributed to ion exchange with Ca^{2+} and Mg^{2+} ions in the SBF composition. Different release rates for different ions also determine a composition of the dissolved material differentfrom that of the initial solid, i.e. an incongruent dissolution of the hydroxyapatites. In simulated dynamic conditions, the linearity of the relationship (2) for days 1-7 is very good; all coefficients of determination are over 0.95, most of them over 0.99.

The results show the importance of diffusion in the release of ions from multisubstituted HAPs, while the calculated kinetic parameters reveal the peculiarities in the release of different ions.

Chapter 5 [6] investigates the *Antibacterial activity of silver nanoparticles obtained by co-reduction with sodium citrateand tannic acid.* Nanomaterials are developed which, added in coatings, have antimicrobial effects. For this purpose, silver nanoparticles were synthesized by a "green" method: co-reduction with sodium citrate and tannic acid, and their antimicrobial action on *Escherichia coli* cultures was investigated. This simple and fast one-pot synthesis led to AgNPs, with controlled size, from 30 to 10 nm, as shown by STEM and AFM images. The presence of elemental silver is evident from the UV-VIs spectra and from the EDX spectra and element distribution maps (Figure 5).

Co-reduction of silver nitrate with sodium citrate and tannic acid at various Ag/TSC/ TA molar ratios was done by heating to boiling the $AgNO_3$ solution and adding the calculated amount of TSC and TA mixture. The solutions were kept boiling under continuous stirring for 15 min. The colloidal silver solution obtained by complete reduction of Ag^+ to Ag had a concentration of 1 mM Ag for the Ag- TSC-TA molar ratio of 1: 7: 2 and 0.25 mM for the molar ratios 1: 7: 0.2, 1: 3: 0.2 and 1: 20: 0.1. The Jasco UV / Vis V650 spectrophotometer was used for UV-VIS absorption spectrum measurements, in the wavelength range from 800 to 190 nm. STEM is a combination of the Hitachi HD-2700 scanning electron microscope (SEM) and the transmission electron microscope (TEM), which operates at a maximum acceleration voltage of 200 kV. STEM is equipped with an Energy Dispersive X-ray Spectrometer (EDS), which has two EDX detectors from Oxford Instruments. STEM-EDS equipment was also used for elemental EDX analysis.

In energy dispersive X-ray spectroscopy, the EDX spectra of the nanoparticles obtained on STEM images (as in the example shown in Figure 5a, b) the presence of Ag as well as of elements from the organic compounds is highlighted (Figure 5b). In Figure 5c-f, the correponding multicolor distribution maps for the individual elements (C, N, O, Ag) can be seen. The distribution maps confirm the presence of Ag mainly in the nanoparticles, while the other elements are distributed over the

entire scanned surface, due to the presence of organic compounds (TSC, TA and their oxidation products) around the particles and between them, being adsorbed on the STEM grid from the deposited colloidal solution.

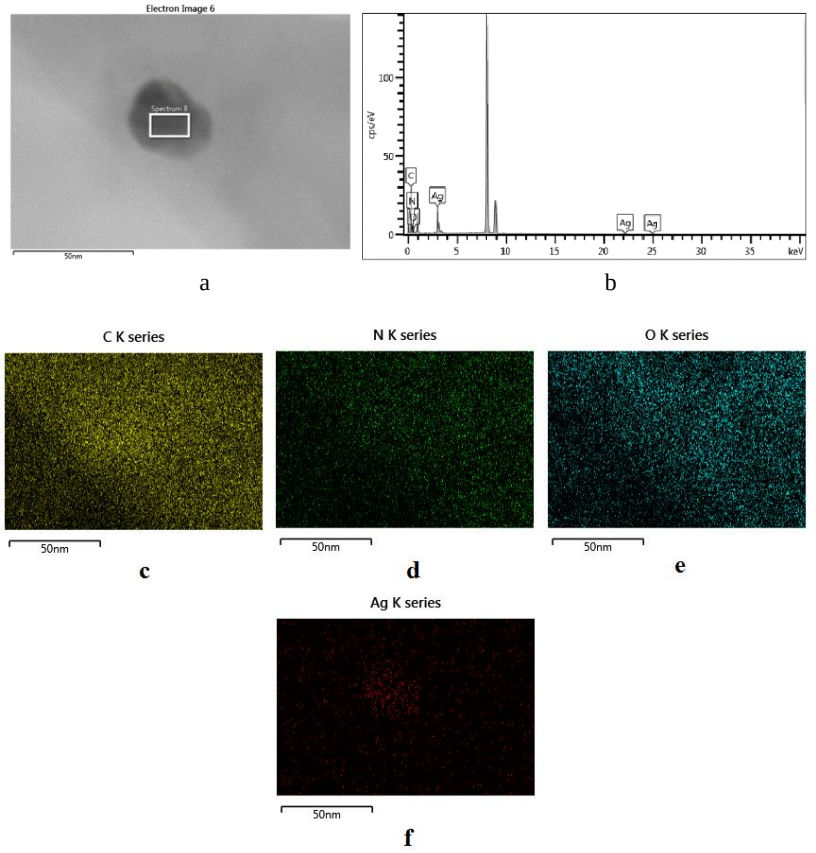

Figure 5. Electronic image (a) and EDX spectrum (b), for the silver nanoparticle (in the white frame, shown in panel a), obtained for sample Ag: TSC: TA with a molar ratio of 1: 3: 0.2; distribution maps for C (c), N (d), O (e) and Ag (f); the bars in the images are 50 nm.

The results of antibacterial testing have shown that, in order to increase the antibacterial activity of AgNPs, various reaction parameters should be considered, including the molar ratio between reducing agent (TSC) and stabilizing (coating) agent, TA of the nanoparticle which seems to be a crucial factor, to finally produce stable nanoparticles of different sizes, preferably with small average diameters. Our results confirm the higher antibacterial activity of the smaller

particles. This effect can be explained by the theory which assigns the antibacterial effect to Ag nanoparticles, because smaller particles have a larger specific surface area, being able to interact more strongly with the cell membrane or can penetrate the cell. On the other hand, if the antibacterial effect were due to Ag^+ ions, there is again the larger specific surface area the one that would guarantee a more intense release of silver ions. To these reasonings we could add that, for the diffusion in agar plates, which is slower than in liquid medium, the smaller particles have a higher mobility, thus increasing the inhibition zones.

Chapter 6 focuses on *the Interaction of silver nanoparticles withvancomycin: an UV-VIS study* [8]. To enhance the antibacterial effect of AgNP, but also of antibiotics, against which bacteria develop resistance, the possibility of using tigether AgNPs and antibiotics, for example vancomycin, was studied. For this purpose, the interaction with vancomycin of AgNPs prepared with different reducing and coating agents was thoroughly investigated: trisodium citrate, β-cyclodextrin, glucose-starch mixture (Figure3), glucose-TEOS (Figure. 4), citric-tannic acid in various ratios. This knowledge of the characteristic behavior of vancomycin AgNP systems can help select the appropriate systems to maximize their antimicrobial effect.

For some preparations (e.g. AgNPs-citrate or AgNPs-citrate-tannicacid) stable AgNPs-vancomycin associations (complexes) were obtained. Forother colloidal solutions (e.g. AgNPs-βCD) less or more advanced vancomycinmediated self-assemblies of AgNPs appeared, and still maintained in colloidalsolution. Finally, in other systems (e.g. AgNPs-glucose-starch or AgNPs-glucose-TEOS), the AgNPs slightly precipitated under the influence of vancomycin.

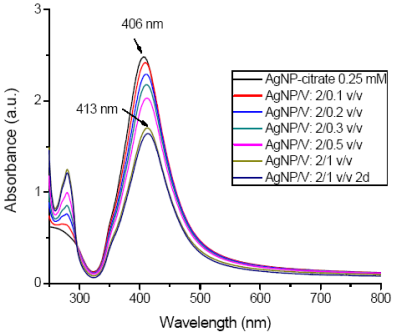

Figure 1. UV-VIS spectra of AgNPs-citrateand V solutions in different ratios and in time

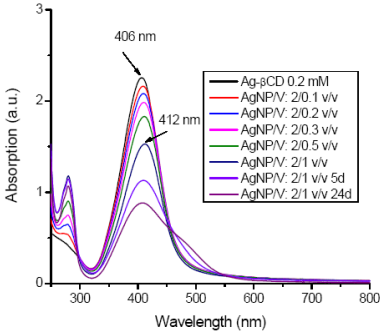

Figure 2. UV-VIS spectra of AgNPs-β-cyclodextrin (β-CD) and V solutions in different ratios and in time

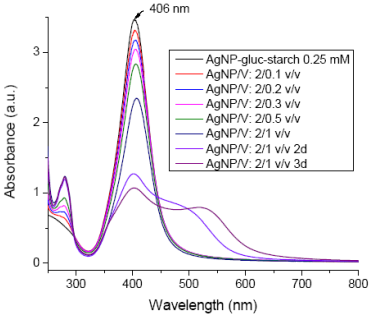

Figure 3.UV-VIS spectraof AgNPs-glucose-starch (gluc-starch) and V solutions in different ratios

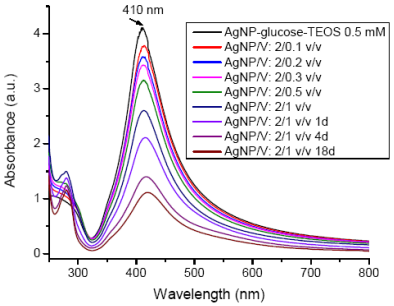

Figure 4.UV-VIS spectra of AgNPs-glucose-TEOS and V solutions and in time indifferent ratios and in time

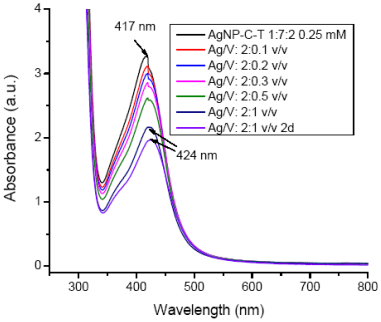

Figure 5. UV-VIS spectra of AgNPs-citrate (C) and tannicacid (T) in the molar ratio 1:7:2 and V solutions in different ratios and in time

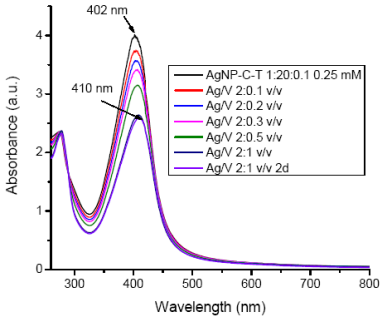

Figure 6. UV-VIS spectra of AgNPs-citrate (C) and tannic acid (T) in the molar ratio1:20:0.1 and V solutions in different ratios and in time

Chapter 7 aims to research *Biomimetic nanocomposite structures designed for the coating of orthopedic implants: AFM investigation* [11].The complex coatings made on Ti implants, containing multisubstituted HAPs, were subjected to a detailed study by AFM, following all the stages of material deposition on the Ti surface, from the preparation of the metal surface, to the formation of collagen fibers on the porous composite (Figures 5, 8, 12). The AFM study was supplemented by XRD investigations (Figure 6).

Ti rods were tested by X-ray diffraction (XRD). The obtained pattern evidences the diffraction peaks only for titanium proving the highest purity of Ti rods.

The rounded titanium rods were flattened with a hydraulic press and cut into sticks with a 20 mm length Both sides of the sticks were grinded with P500

abrasive paper for 10 minutes to obtain a proper texture of the active surface. The grinding debris was removed by intense washing with bi-distilled water, followed by an ultrasound cleaning. After cleaning the rods were chemically activated for 30 min with orto-phosphoric acid to obtain a perfectly clean and degreased surface.

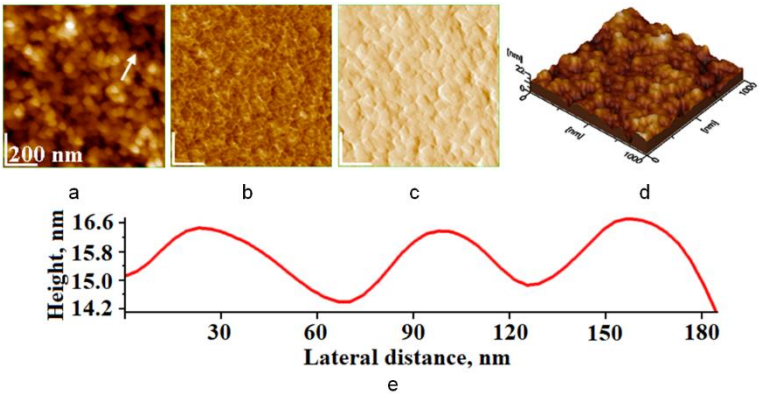

Figure 5. AFM images of HAPc-6%COL (core-shell) nanoparticles: a) topographic image, b) phase image, c) amplitude image, d) 3D image, and e) profile along the arrow in panel (a). Scanned area 1 μm x 1 μm; Ra 2.29 nm; Rq 2.89 nm.

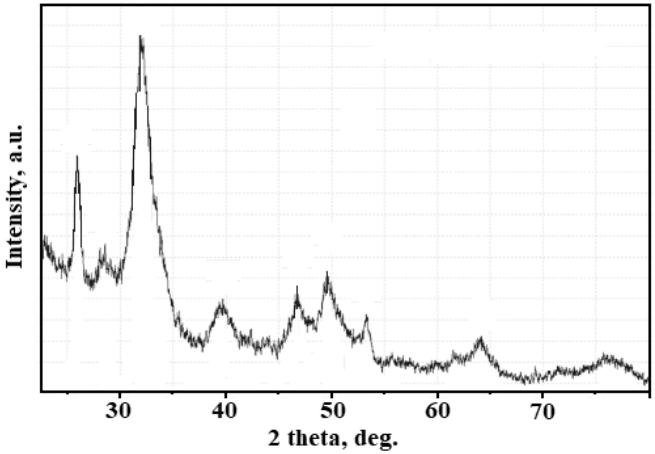

Figure 6. XRD pattern for HAPc-6%COL (core-shell) nanoparticles

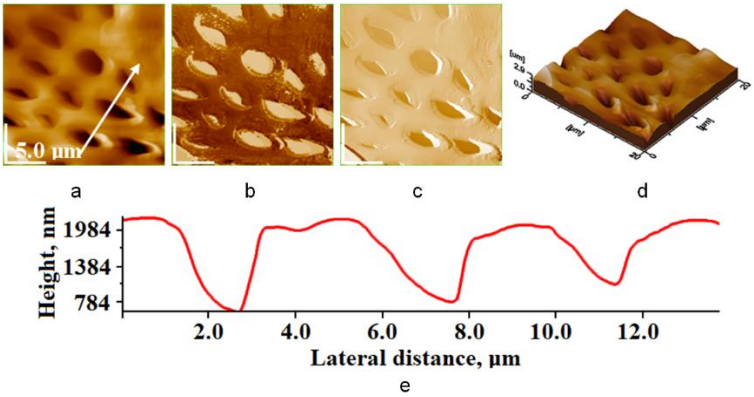

Figure 8. AFM images of pores network on HAPc-6%COL@PLA surface: a) topographic image, b) phase image, c) amplitude image, d) 3D image, and e) profile along the arrow in panel (a). Scanned area 20 µm x 20 µm; Ra 289 nm; Rq 360 nm.

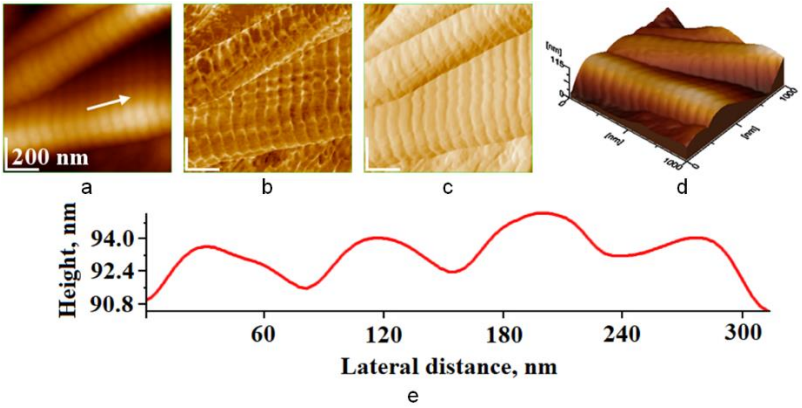

Figure 12. AFM images of the HAPc-6%COL@PLA/COLcoating on Ti implant: a) topographic image, b) phase image, c) amplitude image, d) 3D image, and e) profile along the arrow in panel (a). Scanned area 1 µm x 1 µm; Ra 16.2 nm; Rq 18.2 nm

The major component in ther coating material is the freeze dried ms-HAP/6%COL core-shell NPs. The HAPc NPs were investigated by AFM, and appear well individualized particles adsorbed on the Ti surface (Figure 5).

The rod coating dispersions contained HAPcfunctionalized with 6% collagenin a solution of PLA in dichloromethane. The fluidity of this dispersion is

increased by the addition of acetone. The titanium surface prepared as described above was coated with successive layers, using the dip-coating method. A layer of pure collagen was transferred by vertical adsorption for 5 seconds from a solution rich in COL at pH 12, resulting in the biomimetic structure HAPc-6%COL@PLA/COL on the Ti implant.

Porosity is an important requirement for a bone biomimetic structure to assure enough space for osteoblasts adhesion and proliferation. The pores network is a useful structure as observed in Figure 8, for HAPc-6%COL@PLA coating on Ti implant. These pores are of submicron size and are lastly generate by the slower evaporation of dichloromethane (DCM) under drying process of biocomposite.

On the surface of the HAPc-6%COL@PLA/COL coating, the AFM images show the formation of collagen fibers by self-assembly (Figure 12).

Besides qualitative characterization, AFM image processing allowed quantitative determinations, such as measuring surface roughness, an important feature for their physical and biological role (Tables 1, 2).

Table 1. The surface roughness, Ra and Rq (RMS), of Ti implants before coatings, evaluated by AFM.

Ti	Ti Cold pressed		Ti Grinded		Ti Grinded and etched with acid	
Fig.	Ra±SD nm	RMS±SD nm	Ra±SD nm	RMS±SD nm	Ra±SD nm	RMS±SD nm
2	265±25	330±30	-	-	-	-
3	-	-	154±17	186±19	-	-
4	-	-	-	-	176±18	218±20

Table 2. The surface roughness, Ra and Rq (RMS), of biomimetic coatings on Ti implants measured by AFM

Composite	HAPc-6%COL@PLA		HAPc-6%COL@PLA/COL	
Fig.	Ra±SD nm	RMS±SD nm	Ra±SD nm	RMS±SD nm
8	289±24	360±27	-	-
10	-	-	256±15	304±26

Chapter 8
GENERAL CONCLUSIONS

1). In the research of the doctoral thesis, we aimed to develop biomimetic composites for coating Ti implants (**Chapter 1**). The biomimetic coating (structure) was successfully developed using multisubstituted hydroxyapatite (ms-HAP) functionalized with collagen, COL (i.e. ms-HAP/COL nanoparticles core/shell), embedded in polylactic acid matrix (PLA), resulting in a porous biomimetic structure ms-HAP/COL@PLA, and subsequently coated with a self-assembled layer of COL fibers, obtaining a fibrous biomimetic composite ms-HAP/COL@PLA/COL, called HAPc. Subsequently, these implants were tested *in vivo* to assess bone consolidation in the absence or presence of high frequency pulsed electromagnetic waves (HF-PESW)

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2). For *in vivo* evaluation, albino rats divided into four groups were used: control group (CG) with Ti implant; PESW group with Ti + HF-PESW implant; HAPc group with Ti implant coated with HAPc; HAPc + PESW group with Ti implant coated with HAPc + HF-PESW. The left femoral diaphysiswas fractured and fixed intramedullary. From the first postoperative day, the PESW and HAPc + PESW groups underwent HF-PESW stimulation for 14 consecutive days. The biomimetic coating was characterized by XRD, HR-TEM, SEM, EDX and AFM.

3) The use of HAPc-coated Ti implants together with HF-PESW stimulation positively influenced the bone consolidation process, especially in its early phase.

4). This *in vivo* evaluation demonstrated that the association between HF-PESW stimulation and HAPc coating on Ti implants promotes an accelerated healing process of bone fracture, enhancing bone consolidation in its early phase. Consequently, this combined method is potentially interesting and useful for clinical applications, proving a superior approach to the surface modification of biomedical implants.

5). The biocompatibility of uncovered titanium, Ti, nails, and coated with an innovative biocomposite was also assessed on a rat model of femoral fracture (**Chapter 2**). The biocomposite is based on multisubstituted hydroxyapatite, ms-HAP, containing Mg, Zn and Si, and is used as a coating material deposited on Ti implants, due to the excellent biocompatibility and osteoconductive property of ms-HAP.

6). Intramedullary titanium nails coated with multisubstituted hydroxyapatite and collagen in a polylactic acid matrix stimulate bone healing and also increased implant osseointegration into the host bone. In the case of clinical application of these implants, they could reduce the risk of implant default.

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7). In order to clarify the contribution of multisubstituted hydroxyapatites to the delvery of ions with an important biochemical role, the behavior of multisubstituted hydroxyapatites in water and simulated body fluid, SBF, was studied (**Chapter 3**).

8).The introduction of essential elements with important biological effects in nanostructured hydroxyapatite has been demonstrated by physico-chemical investigations. Multisubstituted hydroxyapatites showed an average degree of crystallinity and a particle size in the nanometric range. The presence of Mg and Zn has a destabilizing effect on the HAP network, while the addition of Sr diminishes this effect.

9). The release of the component elements of these multisubstituted hydroxyapatites was examined in aqueous solutions, as a model for biological fluids. The increased concentration of Sr in the HAP structure significantly influenced the release of Sr in both environments: water and simulated body fluid.

10). A different profile for Ca, Mg and P is determined in both environments, and the formation of a new biomimetic hydroxyapatite is apparently promoted in SBF.

11). The sustained release observed for thevaluable physiological elements contained in multisubstituted hydroxyapatites reveals a promising future for these biomaterials for biomedical purposes.

12). The release of ions from multisubstituted hydroxyapatites was interpreted on the basis of a theoretical model (Higuchi model) in **Chapter 4**.

13). Considering the validity ranges of the Higuchi model for the ions release from the investigated HAPs, we can affirm that, while diffusion is important throughout the entire process of ion release in static conditions, from day 1 to 90, dissolution has also a significant contribution in the initial phase of the process. After the dissolution of the outer, more soluble, shell of particles, the internal diffusion of ions from the bulk to the interface with the immersion medium will be the main process. Moreover, in time a saturation of the solution is approached due to the low solubility, so diffusion remains predominant.

14). In simulated dynamic condition, when the immersion liquid is daily renewed, no saturation could occur, so both dissolution and diffusion contribute to the ion release.

15). To enhance the antimicrobial activity of hydroxyapatites, they can be combined (doped) with substances with antimicrobial action, such as silver nanoparticles or various antibiotics.

16). The characterization of silver nanoparticles obtained by co-reduction with sodium citrate and tannic acid was performed by UV-VS spectroscopy, scanning transmission electron microscopy (STEM) and theywere evaluated by atomic force

microscopy (AFM) images. Their antibacterial effect was highlighted against *Escherichia coli* (**Chapter 5**).

17). Co-reduction of silver nitrate in the aqueous solution with TSC and TA proved to be a simple and fast one-pot synthesis to prepare AgNPs, with controlled size, from 30 to 10 nm, as shown in STEM and AFM images. . The presence of elemental silver is evident from the UV-VIS spectra (SPR characteristic band) and from the EDX spectra and element distribution maps. The antibacterial effect of AgNP was tested by measuring areas of inhibition on *Escherichia coli* cultures.

18). The effect was obvious for all AgNPs samples, but the dispersions with the smallest particle size proved to be the most active. Thus, AgNPs obtained by the investigated methods could be successfully used, as such or in combination with antibiotics, against bacterial infections.

19). The antimicrobial activity of antibiotics can be potentiated by using them together with silver nanoparticles. The interaction of such an antibiotic, vancomycin with silver nanoparticles was studied by the UV-VIS method (**Chapter 6**).

20). UV-VIS measurements allowed us to evence the different behavior of colloidal AgNPs solutions, obtained using different reducing systems, due to the formation of AgNPs with different coating (stabilization) agents. Furthermore, the interaction of these AgNPs with antibiotics, such as vancomycin, is identified.

21). For some preparations (e.g. AgNPs-citrate or AgNPs-citrate-tannicacid) stable AgNPs-vancomycin associations (complexes) were obtained. Forother colloidal solutions (e.g. AgNPs-βCD) less or more advanced vancomycinmediated self-assemblies of AgNPs appeared, and still maintained in colloidalsolution. Finally, in other systems (e.g. AgNPs-glucose-starch or AgNPs-glucose-TEOS), the AgNPs slightly precipitated under the influence of vancomycin.

22). The developing of biomimetic coating onto the Ti surface proves to be a smart choice to enhance the osseointegration and ensure an optimal healing process, due to the creation of nanostructured biomaterials similar to those in native bone. Thus, we designed a composite coating based on multi-substituted hydroxyapatite (noted ms-HAP or HAPc) nanoparticles, NPs, doped with essential elements: Mg, Zn and Si, functionalized with collagen type 1 (COL), embedded into poly lactic acid, PLA, matrix, and finally covered with COL layer to achieve biomimetic structures. Thin layers of biomimetic composite were self-assembled onto Ti surface via dip-coating method. Both, initial and coated Ti implants were investigated by atomic force microscopy (AFM), which allows surface investigation at high resolution of nano-level (**Chapter 7**).

23). AFM is a powerful tool for investigation of the biomimetic composite coating on the titanium surface of implants. It proves that the collagen amount in the nano-composite material is able to reticulate, as COL fibers. The AFM images revealed a

biomimetic network of collagen fibers similar to the one in natural bone formed on the surface of nano-composite layers.

24). The nano-topography and surface roughness are evidenced by AFM microscopy in the coating layers on Ti implants and are suitable for osteoblasts attachment to the surface increasing the cells viability and prolifertion.

25). Adding an extra layer of pure collagen on the coatig layers could be a model of enhancingthe osteoblasts activity to generate new bone on the revealed biomimetic structures.

List of Research Papers- Original Contribution **(Reka Balint)**

8 Articles were published in scientific journals ISI, **Cumulative impact factor: 8.515; 3 articles are accepted** for publication **(*in press*)** in scientific journals ISI;
2 BDI articles and one article in Proceedings

1. P. T. Frangopol, A. Mocanu, V. Almasan, C. Garbo, **R. Balint**, G. Borodi, I. Bratu, O. Horovitz, M. Tomoaia-Cotisel, Synthesis and structural characterization of strontium substituted hydroxyapatites, Revue Roumaine de Chimie, 61(4-5), 337-344, (2016). IF = 0.304.

2. F. Goga, E. Forizs, G. Borodi, Gh. Tomoaia, A. Avram, **R. Balint**, A. Mocanu, O. Horovitz, M. Tomoaia Cotisel, Behavior of doped hydroxyapatites during the heat treatment, Rev. Chim.(Bucharest), 68(12), 2907-2913, (2017). IF = 1.412.

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4. A. Mocanu, **R. Balint**, C. Garbo, L. Timis, I. Petean, O. Horovitz, M. Tomoaia-Cotisel , Low crystallinity nanohydroxyapatite prepared at room temperature, Studia UBB Chemia, 62(2), Tom I, 95-103, (2017). IF = 0.305.

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6. S. Rapuntean, **R. Balint**, G. A. Paltinean, G. Tomoaia, A. Mocanu, C.-P. Racz, O. Horovitz, M. Tomoaia-Cotisel, Antibacterial activity of silver nanoparticles obtained by co-reduction with sodium citrate and tannic acid, Studia UBB Chemia, 63(3), 73-85, (2018). IF = 0.275.

7. D. Oltean-Dan, G.-B. Dogaru, M. Tomoaia-Cotisel, D. Apostu, A. Mester, H.-R.-C. Benea, M.-G. Paiusan, E.-M. Jianu, A. Mocanu, **R. Balint**, O.-C. Popa, C. Berce, G.-I. Bodizs, A.-M. Toader, Gh. Tomoaia, Enhancement of bone consolidation using high frequency pulsed electromagnetic short-waves and titanium implants coated with biomimetic composite embedded into PLA matrix: in vivo evaluation, Int J Nanomedicine, 14, 5799–5816, (2019). IF = 5.115.

8. **R. Balint**, G. A. Paltinean, A. Mocanu, O. Horovitz, M. Tomoaia-Cotisel, Interaction of silver nanoparticles with vancomycin: an UV-VIS study, Studia UBB Chemia, 64(2), Tom II, 335-343, (2019). IF = 0.494.

9. D. Oltean-Dan, P.T. Frangopol, **R. Balint**, Gh.Tomoaia, A. Mocanu, M. Tomoaia-Cotisel, Biocompatibility of titanium implants coated with biocomposite in a rat model of femoral fracture, Studia UBB Chemia, 66(3), (2021). *In press*.

10. A. Mocanu, P. T. Frangopol, **R. Balint**, O. Cadar, I. Vancea, R. Mantiu, O. Horovitz, M. Tomoaia-Cotisel, Higuchi model applied to ions release rate from hydroxyapatites, Studia UBB Chemia, 66(3), (2021). *In press*.

11. **R. Balint**, I. Petean, P.T. Frangopol, A. Mocanu, G. Arghir, O. Horovitz, M. Tomoaia-Cotisel, Biomimetic nanocomposite structures designed to cover orthopedic implants: AFM research, Studia UBB Chemia, 66(3), (2021). *In press.*

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14. **R. Balint**, M. Tomoaia-Cotisel, Gh. Tomoaia, "Compozite biomimetice avansate utilizate în căptusirea implantelor metalice pentru vindecarea fracturilor osoase", Academia oamenilor de stiintă din România (AOSR), Conferinta de toamna, "Convergenta reala Romania-Uniunea Europeana, CRUE", Comunicari integrale, Vol. II, Brasov, 20-21 Septembrie, pages 266-274, (2019), ISBN 978-973-618-430-

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Chapter 10. ORIGINAL SCIENTIFIC RESEARCH

List of Original Research; Original Contributions- Reka BALINT (pag. 25)

8 Articles were published in scientific journals ISI, **Cumulative impact factor: 8.515; 3 articles are accepted** for publication in scientific journals ISI **(*in press*)**; **2** BDI articles and **one** article in Proceedings

Total citations: 60, h-index 5; Google Scholar profile:
https://scholar.google.com/citations?user=SkRatYcAAAAJ&hl=ro

List of communications at conferences and symposia
The scientific results were presented at 13 conferences

1. C. Garbo, A. Mocanu, V. Almasan, **R. Balint**, G. Borodi, I. Bratu. O. Horovitz, M. Tomoaia-Cotisel, Effect of preparation conditionsand the presence of surfactant on the hydroxyapatite nanopowders, PIM 2015, September 23-25, 2015

2. A. Danistean, C. Garbo, **R. Balint**, G. Tomoaia, A. Mocanu, S. Rapuntean, O. Horovitz, M. Tomoaia-Cotisel, Bioceramics comprising silver nanoparticles, as a

new generation of antimicrobial, Conferences on Physics of Advanced Materials, Cluj-Napoca, 8-10 September, 2016

3. **R. Balint**, C. Garbo, A. Mocanu, G. Tomoaia, I. Petean, O. Horovitz, M. Tomoaia-Cotisel,Revolving research discoveries into health, COST Action MP1301, Cluj-Napoca, 13-15 May, 2017

4. **R. Balint**, G. Tomoaia, S. Rapuntean, A. Mocanu, O. Horovitz and M. Tomoaia-Cotisel, Novel composites based on nanoceramics and silver nanoparticles with antimicrobial activity for biomedical applications, International Conference on Materials Science & Materials Chemistry, Paris, France, 20-22 August 2018 **ORAL PRESENTATION + POSTER**

5. D. Oltean-Dan, D. Apostu, G. B. Dogaru, G. Tomoaia, M. Tomoaia-Cotisel, A. Mester, M.-G. Paiusan, A. Mocanu, **R. Balint,** C. O. Popa, C. Berce, B. Gyorgy-Istvan, A. Toader, H.-R.-C. Benea, High frequency pulsed electromagnetic short-waves and titanium nails coated with multi-substituted hydroxyapatite functionalized with collagen embedded intro PLA martri facilitates bone consolidation: an experimenta study, Abstract Volume of 8th International Conference „Biomaterials, Tissue Engineering and Medica Devices" (Biommedd' 2018) Cluj-Napoca, 27-29 September, 2018 **ORAL PRESENTATION**

6. **R. Balint,** M. Tomoaia-Cotisel, G. Tomoaia, "Compozite biomimetice avansate utilizate în căptușirea implantelor metalice pentru vindecarea fracturilor osoase", (Advanced biomimetic compositesused in coating metal implants for healing bone fractures) Conferință națională științifică Academia Oamenilor de Știința din România, Book of abstract, **13**(2), 2019, **20-21 Septembrie Brasov, 80-81, ISSN 2601-5102 ORAL PRESENTATION**

7. **R Balint,** S Rapuntean, A Mocanu, O Horovitz and M Tomoaia-Cotisel,New antibacterial systems for biomedical applications,12th International Conference Processes in Isotopes and Molecules" (PIM), 25-27 September, 2019, Cluj-Napoca, Romania. **POSTER**

8. G. A. Paltinean, **R. Balint**, A. Mocanu, Gh. Rapuntean, I. Petean, O. Horovitz and M. Tomoaia-Cotisel, Antimicrobial activity of poly lactic acid microspheres loaded with vancomicin, 12th International Conference, Processes in Isotopes and Molecules (PIM), 25-27, September2019, Cluj-Napoca, Romania **POSTER**

9**.** Gh. Tomoaia, **R. Balint**, A. Mocanu,M. Tomoaia-Cotisel,Captuseli/straturi bioactive pe implante din titan (Bioactive coatings/layers ontitanium implants), Conferinta Nationala Stiintifica Academia Oamenilor de Stiinta din Romania, Secție Științe Biologice, 28 May-7 June 2020, **online**

10. Gh. Tomoaia, **R. Balint**,A. Mocanu,M. Tomoaia-Cotisel,Căptuşeli/straturi bioactive pe implante din titan (Bioactive coatings/layers ontitanium implants),, Conferinta Nationala Stiintifica Academia Oamenilor de Stiinta din Romania,Secție Științe Medicale, 18 iunie 2020, **online**

11.Gh. Tomoaia, **R. Balint**, A. Mocanu,M. Tomoaia-Cotisel, Materiale inovative pentru substitut de os (Innovative materials for bone substitutes),Conferinta Nationala Stiintifica Academia Oamenilor de Stiinta din Romania,Sesiunea Științifică de Toamna, Secție Științe Biologice, 27-28 November 2020, online

12. **R. Balint,** Gh. Tomoaia, D. Oltean-Dan, A. Mocanu, G. Arghir, M. Tomoaia-Cotisel,Nanomateriale cu funcție biologică îmbunătățită pentru regenerare osoasă (Nanomaterials with improved biological function for bone regeneration), Conferinta Nationala Stiintifica Academia Oamenilor de Stiinta din Romania, Secție Științe Biologice, 11 June 2021, **online**

13. **R. Balint**, Gh. Tomoaia, D. Oltean-Dan, A. Mocanu, M. Tomoaia-Cotisel, Biomaterials based on multifunctional hydroxyapatite for orthopedic applications, International Scientific Conference, Applications of chemistry in nanosciences and biomaterials engineering, Virtual Conference, 25 - 26 June 2021

Activity in research grants

The doctoral student has worked as a research assistant in **6 scientific research projects** carried out in the Center for Scientific Research in Physical Chemistry, CECHIF, under the supervision of Univ. Prof. Dr. Maria Tomoaia-Cotișel, at Faculty of Chemistry and Chemical Engineering, Babeș-Bolyai University of Cluj-Napoca.

List of scientific grants

1. **PN2 Grant Partnerships 241/2014-2016**

2. **Grant Exploratory Research Project: PCE 83/2017-2019**

3. **Grant Experimental Demonstrative PNIII: Partnerships 481/2020-2022**

4. **Exploratory Research Project: PCE186/2021-2023**

5. **Mobility program for researchers.PN-III-P1-1.1-MC-2018-1737**
   Novel composites based on nanoceramics and silver nanoparticles with antimicrobial activity for biomedical applications; Participation at International Conference on Materials Science and Materials Chemistry 20-22 August, Paris, France. **Responsible for the project: Reka Balint.**

6. Reka Balint: Advanced biomimetic composites awarded in 2019 by the Academy of Romanian Scientists**.**

15.4. Member in the Center of Scientific Research in Physical Chemistry

The doctoral student is a member of the Center of Scientific Research in Physical Chemistry, CECHIF, directed by the Director, Univ. Prof. Dr. Maria Tomoaia-Cotisel, Faculty of Chemistry and Chemical Engineering, Babeș-Bolyai University of Cluj-Napoca.