BABEŞ-BOLYAI UNIVERSITY
FACULTY OF CHEMISTRY AND CHEMICAL ENGINEERING
DEPARTMENT OF CHEMICAL ENGINEERING

PhD THESIS ABSTRACT

RESEARCH AND DEVELOPMENT OF SOME NANOSTRUCTURES OF BIOLOGICAL AND BIOMEDICAL INTEREST

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THESIS SUMMARY

INTRODUCTION

This Ph. D. thesis**, Research and development of some nanostructures of biological and biomedical interest,** belongs to the dynamic multi-disciplinary research area related to nanoparticles and advanced nanostructured biomaterials for biological and biomedical applications. The major objective of this thesis is to develop and implement various nanotechnologies to obtain new nanostructures and nanostructured biomaterials made of nanoparticles of inorganic materials, like synthetic stoichiometric pure hydroxyapatite and multi-substituted hydroxyapatites, doped with different bioactive ions and metallic nanoparticles, like silver nanoparticles, for applications in biology and in biomedicine [**1-4**].

This thesis presents recent original results related to synthesis and characterization of nanoparticulate structures with applications in the field of nanomedicine [**5, 6**]. The synthesis of nanoparticles was conducted by adjusted wet chemistry methods [**5-12**] and their characterization was performed by advanced methods, such as: XRD, FTIR and Raman spectroscopy, TEM, SEM and AFM imaging techniques, by zeta potential and BET determinations as well as by biological and biophysical assays, such as: viability cells (e.g., Alamar Blue and fluorescein diacetate tests), adhesion and proliferation, immunecytochemical staining techniques for cellular protein expression (e.g., osteopontin, osteonectin, collagen type 1, osteocalcin), specific methods for alkaline phoshatase activity, cellular nuclei visualization and adhesion protein (i.e. F-actin) for determination of cellular adhesion.

The thesis begins with this **introduction** and a **state of the knowledge** on nanostructures with biological and biomedical applications.

The present thesis has **eight chapters:** five of them are based on the **8 published articles, 6 ISI articles** [**5-10**] plus **2 articles** [**11, 12**]: one published in Proceedings of ***Granulation Conference*** and another one published in a ***scientific journal*** approved by CNCSIS. Due to the fact that the content of all the 8 chapters are either published as articles or already prepared as manuscript to be submitted to the publication, the content of each chapter follows the sections from those articles and manuscripts.

Chapter 1 is focused on synthesis and maturation processes of pure stoichiometric hydroxyapatites (HAP) of various crystallinity degrees and the characterization of obtained nano scale structures and biomaterials.

Chapter 2 describes the synthesis and physical and chemical characterization of Si-substituted HAP (Si-HAP) as well as the biological performance of osteoblasts in culture on the scaffolds made of these nanoparticles and nanostructures.

Chapter 3 covers the synthesis of porous nanostructures made of Zn substituted hydroxyapatites, Zn-HAP [**7**], and **chapter 4** deals with Sr-HAP nanostructures [**8**], and for their physical and chemical characterization, nanostructured materials having a high impact on bone regeneration and applications in osteoporosis treatment.

Chapter 5 presents the synthesis and the characterization of multi-substituted hydroxyapatite with Au, Ag, and Zn and its nanocomposites [**6**] with different content in silver nanoparticles (AgNP) [**5**]. Chapter 5 deals with in vitro biological characterization of these nanocomposites, covering the diffusion method used for characterizing their effect on different pathogens in culture medium. In this chapter, the antimicrobial effects of these nanostructures, as nanocomposites of HAP-AgNP, against 5 pathogens, are presented and analysed.Chapter 5 also presents the recent research on nanostructures, made of hydroxyapatite, nitroxoline and Ag^{+} ions [**9**], as well as made of hydroxyapatite, nitro-hydroxy-quinoline in the presence of Zn^{2+} ions [**12]**, and indicates the sensibility of various micro-organisms in vitro to these nanocomposites.

Chapter 6 covers the synthesis and physical and chemical characterization of hydroxyapatite multi-substituted with Mg, Zn and Si, HAP-Mg-Zn-Si [**11**], and of hydroxyapatite multi-substituted with Mg, Zn, Sr and Si (HAP-Mg-Zn-Sr-Si), of biomedical interest for bone remodelling and reconstruction.

Chapter 7 presents studies of biocompatibility of these nanostructures made of HAP-Mg-Zn-Sr-Si using human mature osteoblasts derived from iliac crest, applying various biological assays, regarding cellular viability, adhesion and proliferation and the cellular protein expression.

Chapter 8 gives the results obtained on nanostructures made of hydroxyapatite and nano-silver, as a novel bone cement of biomedical interest in orthopaedic field and in dentistry [**10**]. These nanostructures can be used for metal coating to improve the bioactivity of implants in vivo.

This thesis ends with **general conclusions** and with a ***list of original publications and communications at conferences and symposia*** and another ***list of selected references*** cited in the thesis. It is to be mentioned that each chapter contains its cited references.

These **8** chapters present over **50** bioactive nanostructures, known also as bioactive ceramics and biomaterials, made of nanoparticles, predominantly of various hydroxyapatites, as bone minerals and silver nanoparticles as antimicrobials, that can be used as bone substitutes, or as modified bone like minerals and nanocomposites for biological and clinical applications, for bone tissue engineering and bone regeneration. These

nanostructured hydroxyapatites and their composites are further investigated in Physical Chemistry Centre, at Faculty of Chemistry and Chemical Engineering, for metallic implant coatings with biological and biomedical applications.

The original results show that the bioactive nanostructures developed in this PhD thesis can be used to further develop modified multi-functional surfaces that can lead to new strategies for the manufacture, testing, and clinical applications for regenerative medicine.

Applications of obtained nanostructures may also include their uses for replacements for hips, knees, teeth, tendons and ligaments as well as for restorations for periodontal disease and spinal fusion, and also for maxillofacial reconstruction and stabilization of teeth alveolar, after teeth extractions.

Other applications might be focussed on scaffolds, made by coating with these nanostructures and bioactive particulate biomaterials on different material surfaces (e.g., optically polished metallic surfaces or glass surfaces) using layer-by-layer assembled techniques resulting in thin bioactive layers, that can be used in cell culture of osteoblasts or stem cells to investigate cellular bioactivity in vitro and in vivo.

The HAP and multi substituted HAP can be also used as carriers of silver nanoparticles having anti-microbial effect against various pathogens (chapter 5). These ceramic nanostructured biomaterials can be used for coating applications in the medical field such as implants and implanted devices for drug release.

The obtained bioceramics and bioaterials might be used for the reconstruction of diseased or damaged parts of the skeletal system. They can be resorbable (e.g., tricalcium phosphate) and bioactive (e.g., hydroxyapatite, multi-sunstituted hydroxyapatites) or porous ceramics for tissue ingrowth (e.g., hydroxyapatite-coated metals).

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Key words: substituted HAP, antimicrobial, bone cement, bone substitutes

NANOSTRUCTURES OF BIOLOGICAL AND BIOMEDICAL INTEREST. Present state of art

Nowadays, nanostructures made of nanoparticles and advanced nanostructured biomaterials for biological and biomedical applications have become an important research platform. Accordingly, the nanotechnology is developed and implemented to serve this field, with the major goal for treating various diseases, and enhancing the quality of life. Therefore, the development of novel nanostructures and nanostructured biomaterials is crucial for bone substitutes, tissue engineering and tissue regeneration.

Nanostructures based on synthetic hydroxyapatite (HAP) began to be currently used as porous implants, as powders or as films for coating metallic prostheses, in order to produce their bioactive fixation and be clinically relevant.

It is the time to consider an innovative strategy on a more biological base for tissue repair, namely to develop ***new nanostructures*** able to activate cells and genes in the human body, helping the body to heal.

The present ***PhD Thesis*** has as major objective to develop ***innovative nanostructures (biomaterials),*** designed to stimulate specific cellular responses at molecular level. The results obtained show that modifications in the composition and structure of hydroxyapatite, which is the predominant mineral in the bone structure, can induce specific

interactions in the cellular proteins (e.g., integrin), and through this, the cellular adhesion, proliferation and differentiation are activated. Finally, the production and organization of the extracellular matrix can result in the acceleration of bone formation and remodeling, with potential applications in tissue engineering, in orthopedics, dentistry and in the active transport of various active principles

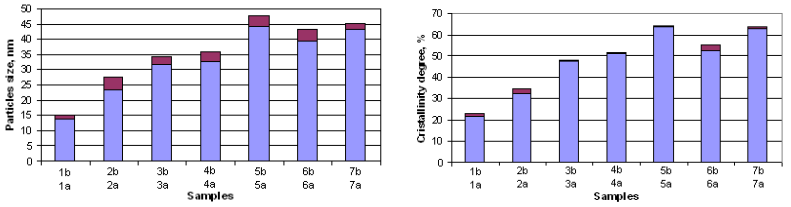
ORIGINAL RESEARCH

1. NANOSTRUCTURES MADE OF HYDROXYAPATITES. Effects of maturation conditions

Hydroxyapatites were prepared by chemical precipitation method from calcium nitrate and diammonium hydrogen phosphate in basic medium. The obtained dispersion was then processed by maturation in various conditions (stages, duration, temperature), resulting in ***seven series of nanostructures*** **(**biomaterials; Fig. 1.2**),** based on hydroxyapatites with different crystallinity degree (Tab. 1.2), and various nanoparticles sizes and shapes (Fig. 1.3). The obtained biomaterials (suspensions, wet precipitates, calcined and not calcined lyophilized powders) were characterized by X rays diffraction (Tab. 1.2), TEM (Fig. 1.3) and AFM imaging, FTIR (Fig. 1.5) and Raman spectroscopy, zeta potential and BET measurements.

Table 2. Crystallite size and crystalinity degree of non calcined (a) and calcined (b) samples of hydroxyapatite lyophilized powders

Sample	1a	1b	2a	2b	3a	3b	4a	4b	5a	5b	6a	6b	7a	7b
Crystallite size (nm)	13.8	15.1	23.3	27.8	31.8	34.5	32.7	35.1	44.0	47.6	39.5	43.2	43.3	45.2
Crystal-linity (%)	21.3	23.1	32.3	34.7	47.5	48.1	51.3	51.7	63.5	64.4	52.5	55.3	63.0	63.5



a. b.

Fig. 1.2. Crystallites size (a) and crystallinity degree (b) for non calcined (blue) and calcined (red) HAP samples

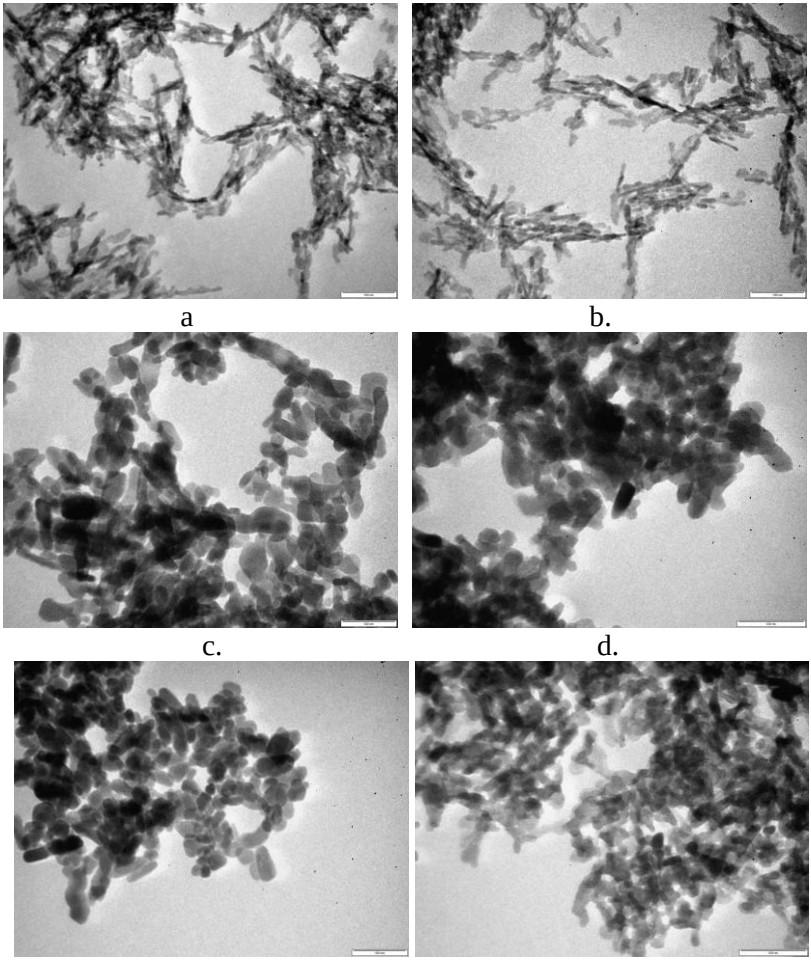
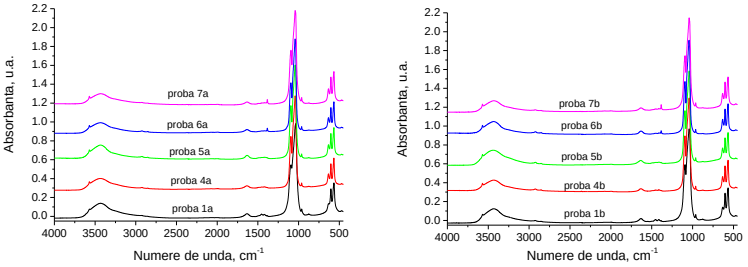


Fig. 1.3. TEM images of hydroxyapatite wet precipitate (paste), dispersed in water: sample 1 (a), sample 2 (b); sample 3 (c), sample 4 (d), sample 5 (e), sample 7 (f); the bars in the images are 100 nm

From Table 1.2 and Fig. 1.2, we observe a general trend for both longer maturation stages and higher temperature of maturation to increase the size of crystallites and the degree of crystallinity. The effect of calcination is an increase of the dimensions of crystallites, but the

crystallinity degree is only slightly affected, particularly for the samples which had a maturation stage at enhanced temperature, 60-80 ^{o}C (samples 4-7).

The FTIR spectra are compared for lyophilized samples without calcination (Fig. 1.5a) and after calcination at 300 ^{o}C for 1 h (Fig. 5b). The spectra were normalized to 1 for the highest absorption peak and shifted along the y axis for comparison.



a. b.

Fig. 1.5. FTIR spectra of lyophilized samples (1, 4, 5, 6, 7) without calcination (a) and after calcination (b); the spectra are normalized

The *BET* results, given as specific surface area, pores specific volume, and most probable pore radii are given in Table 1.5. All the samples present a rather high specific surface area, due both to the high degree of dispersion of the powders, and to their porosity. By calcination the specific surface area is diminished as an effect of nanoparticles sintering, while the pores specific volume is rather unchanged. These physical-chemical; properties are, in turn, critical for the biological activity of the obtained bio-nanomaterials, with a view to their use in biological and medical purposes.

Table 1.5. Some characteristics from BET analysis HAP samples.

Sample	Specific surface area, m^2/g		Pores specific volume, cm^3/g		Most probable pore radius, nm	
	not calcined	calcined	not calcined	calcined	not calcined	calcined
1a, 1b	144.2	90.9	0.332	0.361	7.7	7.7
4a, 4b	106.5	96.3	0.358	0.384	7.7	8.6
5a, 5b	99.9	90.2	0.355	0.378	9.6	8.6
6a, 6b	95.6	86.3	0.358	0.354	7.7	7.7
7a, 7b	78.8	73.6	0.324	0.344	7.7 (11.9)	7.7 (13.3)

2. SILICON DOPED HYDROXYAPATITE NANOSTRUCTURES. Viability and proliferation of osteoblasts *in vitro*

The objective of this research was to investigate the effects of silicate substitution within hydroxyapatite (HAP) lattice on its chemical and physical properties and in vitro osteoblast biological response. A wet chemical method was used to synthesize **four** silicate substituted HAPs (Si-HAP) powders with 0.47 wt%, 2.34 wt%, 4.67 wt% and 9.34 wt% Si, freeze dried and then calcined at 650 ^{o}C for 1h. Biomaterials were investigated by XRD (Fig. 2.1), FTIR and Raman spectroscopy as well as by TEM, SEM and AFM imaging (Fig. 2.9).

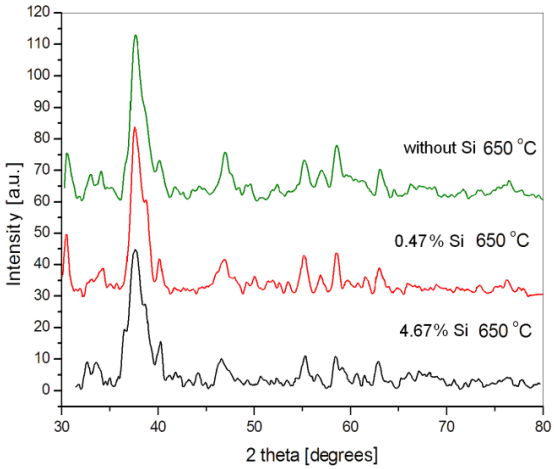


Fig. 2.1. X-ray diffraction patterns for hydroxyapatite, HAP without Si, and HAP with 1% SiO_2 [0.47% Si-HAP)] and HAP with 10% SiO_2 [4.67% Si-HAP] powders, calcined at 650 ^{o}C for 1h.

At low concentration up to 4.67% in Si, a unique phase of HAP substituted with Si is obtained. At higher Si concentration, the role of Si is to promote the formation of two phases, HAP and TCP, each partially substituted with Si. The size of HAP and TCP crystallites was found in the nano crystalline domain, as given in Fig. 2.9.

FTIR and Raman spectra plead for the predominant presence of HAP substituted with silicon at least up to 4.67 mass% in Si. For higher silicon content, silicon was also found as amorphous SiO_2.

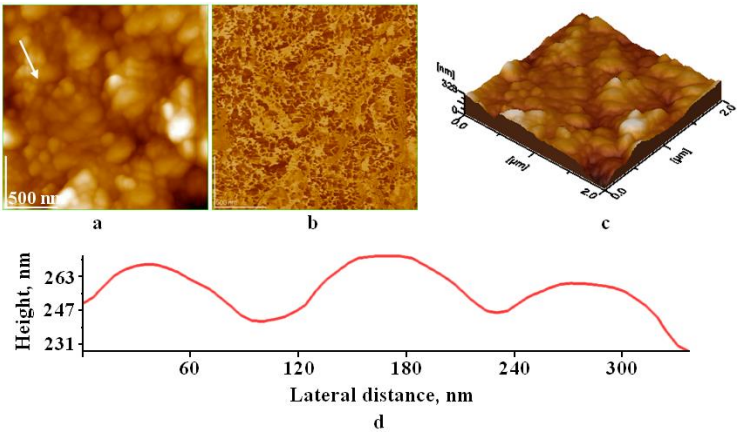


Fig. 2.9. AFM images of Si-HAP with 4.67% Si content, calcined at 650 ^{o}C; scanned area: 2 μm x 2 μm; a) topography image, b) phase image, c) 3D view of topography, d) cross section along the arrow in panel a; Average diameter of nanoparticles of 72 ± 9 nm; RMS on areas is 37.7 nm, RMS on cross profile is 9.12 nm.

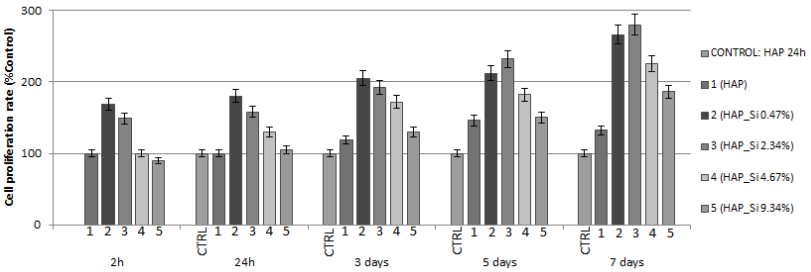


Fig. 2.13. Graphical representation of Alamar blue viability test for osteoblasts harvested on Si-HAP nanostructures, used as scaffolds: pure HAP (1), Si(0.47%)-HAP (2), Si(2.34%)-HAP (3), Si(4.67 %)-HAP (4) and Si(9.34%)-HAP (5). HAP nanostructure at 24h was considered as control (CTRL) for time points: 1, 3, 5 and 7 days of osteoblasts on scaffolds.

These synthesized Si-HAP biomaterials are investigated regarding their influence on cellular adhesion and proliferation (Fig. 2.13) as well as on cell protein expression (Fig. 2.16), in human osteoblasts culture, considering their potential use in bone repair and regeneration.

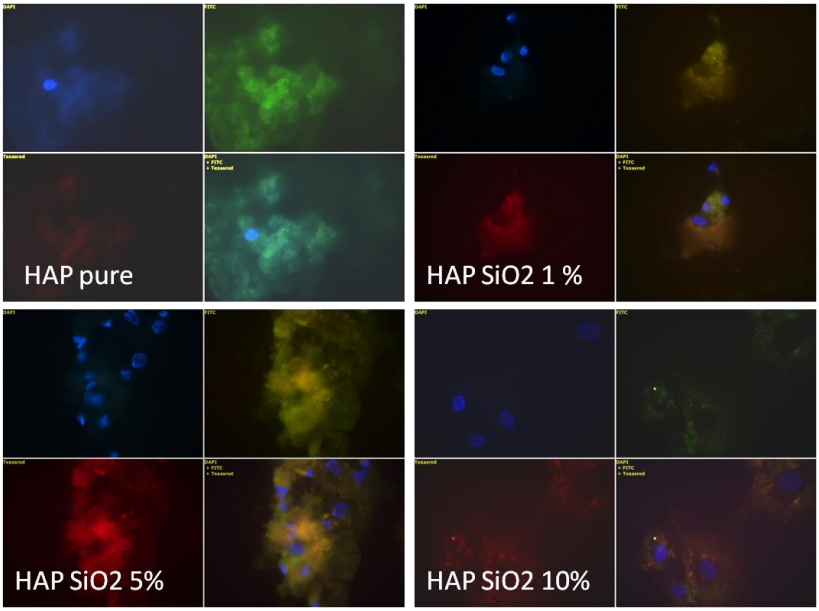


Fig. 2.16. Immunocytochemical staining of osteoblasts: collagen-1, green with FITC, osteocalcine, in red with Texas red, and celular nuclei, bleu with DAPI. Images after 7 days of osteoblasts on scaffolds, made of pure HAP, three biomaterials Si-HAP, with Si content of 0.47% (HAP SiO_2 1%), 2.34% (HAP SiO_2 5%) and 4.67 % (HAP SiO_2 10%).

The scaffolds of Si-HAP nanostructures have a better enhanced activity on osteoblasts than pure HAP (Fig. 2.13) after 1 day. They show a better behavior of osteoblasts than the control formed from pure nano HAP. Clearly, the scaffolds made of HAP-Si(0.47%) and HAP-Si(2.34%) appear to have the best activity from the four nano materials Si-HAP enriched in Si content for proliferation of osteoblasts (Fig. 2.13).

The higher osteoblast adhesion on Si(0.47wt%)-HAP is in agreement with our previous studies, where we demonstrated higher adhesion, at earlier time points (up to 3 days), than the pure HAP [7] and also with the results reported for HAP with Si 0.8 wt% [18], indicating a higher metabolic activity of osteoblasts on these Si-HAP scaffolds. At higher silicon concentrations in HAP, such as Si(2.34%)-HAP, at latter time points (after 5 days), the stimulating effect of Si is higher than for Si(0.47 wt%)- HAP (Fig. 2.13).

This dose dependant effect of biomaterials on bone cells has also been reported for other trace elements. Undoubtedly, there is an optimum concentration of silicon for the stimulation of osteoblasts adhesion, proliferation and differentiation. Consequently, both biomaterials HAP-Si(2.34%) and HAP-Si(0.47%) can be used to stimulate osteoblasts in the formation process of new bone.

The analysis of images given in Fig. 2.16 indicates an advanced organization of collagen type 1 (collagen-1) and of osteocalcin around many cellular nuclei, indicating a high stage of cellular proliferation, particularly induced by the Si(2.34%)-HAP (noted: HAP SiO_2 5%) scaffold in comparison with all others Si-HAP scaffolds, including pure HAP scaffold. These data are in total agreement with alkaline phosphatase activity, and the production of osteopontin and osteonectin, which are the best evidenced in osteoblasts on the Si(2.34%)-HAP scaffold. These results showed that Si-HAP, with 2.34% Si content, significantly enhanced the proliferation, as well as the protein expression, such as osteocalcin, osteopontin, osteonectin, collagen-I and alkaline phosphatase, ALP, of osteoblasts.

These results suggest that Si-HAP biomaterials play an important role in regulating the proliferation and osteogenic differentiation of osteoblasts into osteocytes, as well as in the bone formation.

Definitely, the silicon content within HAP lattice can be related to its effect on the chemical structure, composition and properties and also on the morphology and surface roughness of these biomaterials (bioceramics). These nanobiomaterials are currently used in our laboratories to fabricate scaffolds to be used in osteoblasts culture to evaluate their performances in vitro as a first step to in vivo future studies.

3. NANOSTRUCTURES FROM ZINC DOPED HYDROXYAPATITES

Chapter 3 presents the synthesis of new porous nano-hydroxyapatites (HAP), nanohydroxyapatites substituted with different Zn contents (HAP_Zn), from 0.2 to 10 wt%, conducted in the presence of a surfactant, such as L-asparagine, for controlling the pore size distribution and morphology of obtained nanopowders, using a co-precipitation process. So a new approach was developed for the preparation of HAP-Zn nanopowders with improved nucleation and crystallization ability [**C. Garbo**, M. Sindilaru, A. Carlea, Gh. Tomoaia, V. Almasan, I. Petean, A. Mocanu, O. Horovitz, M. Tomoaia-Cotisel, Synthesis and structural characterization of novel porous zinc substituted nanohydroxyapatite

powders, *Particulate Science and Technology*, DOI:10.1080/02726351.2015.1121180**].**

XRD measurements (Fig. 3.1) indicated HAP as the single phase present and evidenced the nanostructured nature of the obtained HAPs (Fig. 3.6). FTIR spectra also confirmed the presence of the HAP structure. TEM, SEM and AFM images showed the morphology of HAP-Zn nanostructures. BET analysis enabled the characterization of specific surface area and samples porosity. The new nanopowders are designed to be used in orthopedic surgery, particularly for the treatment of osteoporosis, and as bone substitutes, and also in dentistry for enamel remineralization.

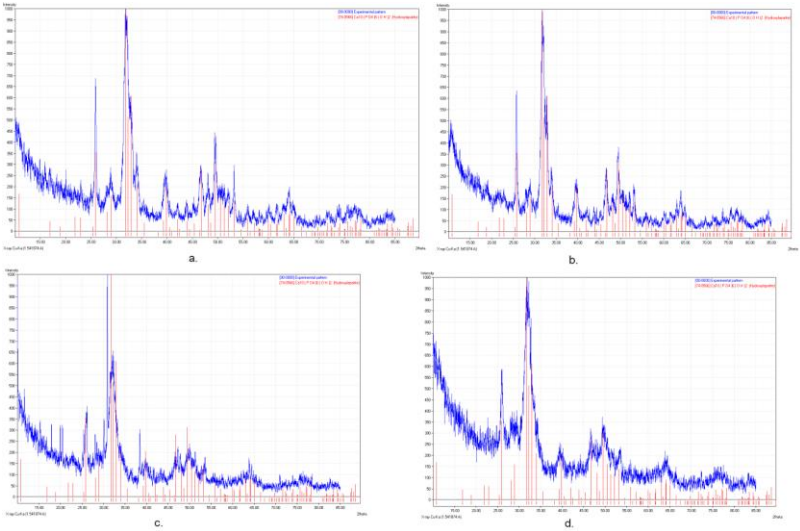


Fig. 3.1. XRD pattern of uncalcined (a) and calcined (b) HAP_0.2%Zn sample, and uncalcined (c) and calcined (d) HAP_10%Zn sample, compared with PDF 74-0566 for stoichiometric hydroxyapatite.

A general trend for decreasing the average size of crystallites with increasing Zn content substituting for Ca is observed. A similar trend is also manifest for the crystallinity degree of HAP-Zn nanopowders.

Generally, the HAP_Zn nano powders are composed of spherical, quasi-spherical or ellipsoidal nanoparticles, ranging from 34 nm to 47 nm (Fig. 3.6). No significant changes are observed in the structure and morphology of thin layers of either calcined or non calcined HAP-Zn powders, in good agreement with TEM images. These results are in

substantial agreement with obtained data on crystallite sizes from XRD patterns.

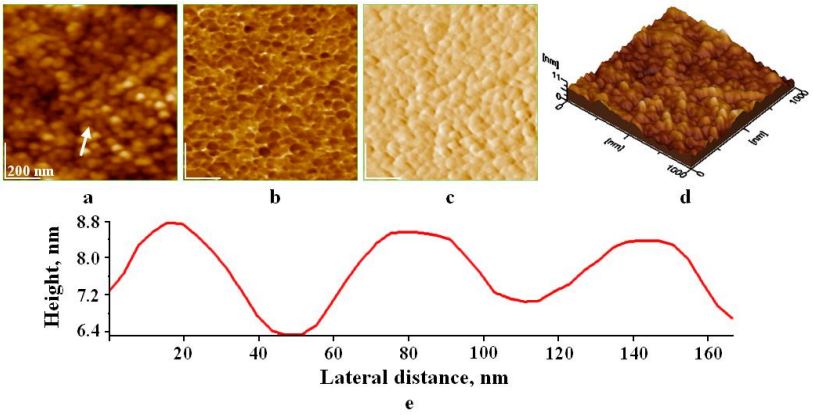


Fig. 3.6. AFM images for HAP-10 % Zn nanoparticles, adsorbed on glass, from aqueous dispersion: a) 2D topography; b) phase image; c) amplitude image; d) 3D topography; e) croos section profile; scanned area 1000 nm × 1000 nm.

4. STRONTIUM DOPED HYDROXYAPATITE NANOSTRUCTURES

Novel strontium substituted nano hydroxyapatites of high crystallinity were prepared by a new wet chemical methodology, assisted by templates (ethylenediamine and o-toluidine), starting with calcium and strontium nitrates and diammonium hydrogen phosphate in stoichiometric ratio, processed in basic medium (ammonia). [P.T. Frangopol, A. Mocanu, V. Almasan, **C. Garbo**, R. Balint, G. Borodi, I. Bratu, O. Horovitz and M. Tomoaia-Cotisel, Synthesis and structural characterization of strontium substituted hydroxyapatites, *Rev. Roum. Chim.*, *61*(4-5), 339-346 (2016)].

Synthesized hydroxyapatites correspond to the formula $Ca_{10-x}Sr_x(PO_4)_6(OH)_2$ with x = 0 (pure hydroxyapatite, HAP), 0.59, 1.21 and 1.87 (HAP-Sr) as well as 10, corresponding to Ca totally substituted by Sr (SrHAP). They were characterized by XRD (Fig. 4.1), FTIR and Raman spectroscopy (Fig. 4.2), as well as by TEM and AFM.

Strong correlations were found between the Sr content, crystallites size and crystallinity for the obtained nanopowders. These nanomaterials showed a controlled morphology and can be used in orthopedics, as

substitutes for bone remodeling, as well as in dentistry, as desensibilization agents and for enamel remineralization.

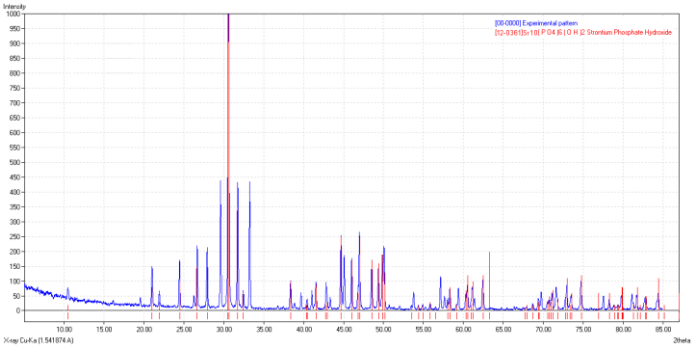


Fig. 4.1. XRD pattern for the SrHAP nanopowder compared with PDF for $Sr_{10}(PO_4)_6(OH)_2$.

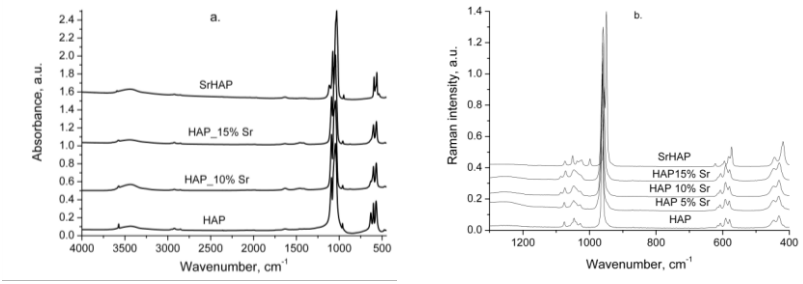


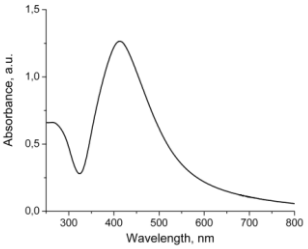
Fig. 4.2. Normalized FTIR spectra (a) and Raman spectra (b) of HAP and Sr-substituted HAPs

Thus, the template mediated wet chemical methodology, reported here, leads to highly crystalline products with controlled nano structure and morphological features. These new HAP-Sr nanomaterials have potential applications in biomedical engineering, especially in orthopedic, dental and maxillofacial field.

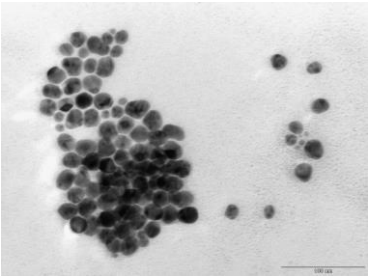
5. NANOSTRUCTURES OF Ag, Au, Zn AND SILVER NANOPARTICLES DOPED HYDROXYAPATITE

Nano hydroxyapatite doped with Ag (0.25 wt%), Au (0.025 wt%), and Zn (0.2 wt%), (HAP-Ag-Au-Zn), has been obtained by an innovative

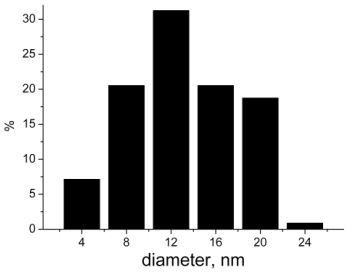
wet chemical approach, coupled with a reduction process for silver and gold. **[**A. Mocanu, G. Furtos, S. Rapuntean, O. Horovitz, C. Flore, **C. Garbo,** A. Danisteanu, Gh. Rapuntean, C. Prejmerean, M. Tomoaia-Cotisel, Synthesis; characterization and antimicrobial effects of composites based on multi-substituted hydroxyapatite and silver nanoparticles, *Applied Surface Science*, **298**, 225–235 (2014)**].** The synthesized multi-substituted nano HAP was freeze-dried and calcined at 650 ^{o}C. Nano HAP has been characterized by XRD, FTIR spectroscopy and imaging techniques: TEM, SEM and AFM.



a.



b.



c.

Fig. 5.1. Characterization of silver nanoparticles (AgNPs): (a) UV-Vis spectrum; (b) TEM image; the bar in the image is 100 nm; (c) histogram of size distribution for AgNPs

Then, nano HAP was mixed with previously synthesized silver nanoparticles (AgNPs), in the amount of 9 wt%, to give a novel material (HAP-Ag). The AgNPs were prepared by the reduction of silver nitrate with glucose in alkaline medium. TEM and UV-Vis confirmed the formation of AgNPs with an average size of 12 nm (Fig. 5.1): **[**A. Mocanu, R.D. Pasca, G. Tomoaia, **C. Garbo**, P.T. Frangopol, O. Horovitz, M.

Tomoaia-Cotisel, New procedure to synthesize silver nanoparticles and their interaction with local anesthetics, Int. J Nanomedicine, 8 (2013) 3867-3874**]**. The presence of AgNPs was also revealed in XRD paterns (Fig. 5.9).

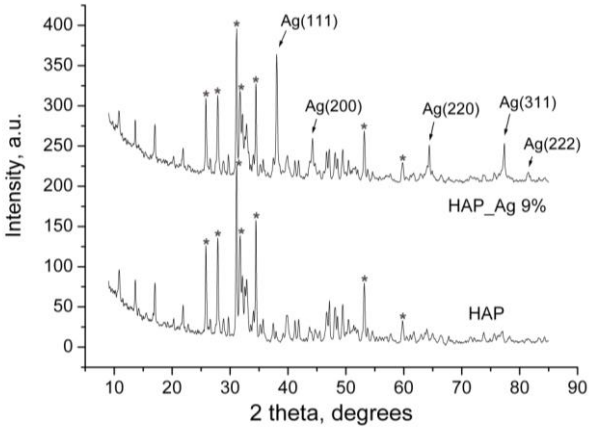


Fig. 5.9. RXD patterns for complex HAP, and complex HAP with 9% Ag adsorbed (characteristic peaks for HAP are marked with *)

Further, organic matrix composites were obtained from HAP-Ag and a mixture of monomers (such as bis-GMA and TEG-DMA), which were polymerized at various compositions in silver content up to 5.4 wt%. The new material HAP-Ag contains AgNPs homogeneously adsorbed on the particles surface of Ag, Au, Zn triple substituted nano-HAP. These composites are thoroughly researched as regards their structure and antimicrobial properties

Antibacterial activities of these composites were investigated against several different pathogenic species: *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus spp.*, *Bacillus cereus*, and *Candida albicans*, using the Kirby-Bauer disk-diffusion method. Antibacterial activities are enhanced with increasing of silver content within composites. (Fig. 5.10, Tab. 3.5).

The inhibitory effect was observed for all the five microbial species tested. Inhibition zone diameter increases simultaneously with the increase in silver content of the samples. The best efficacy was attained for the highest silver contents, 4.5% and 5.4 %. There were not significant differences between readings at 24 and 48 incubation hours. The data show

a significant antimicrobial effect of the slow release of Ag^+ ions *in vitro* against the five pathogenic species.

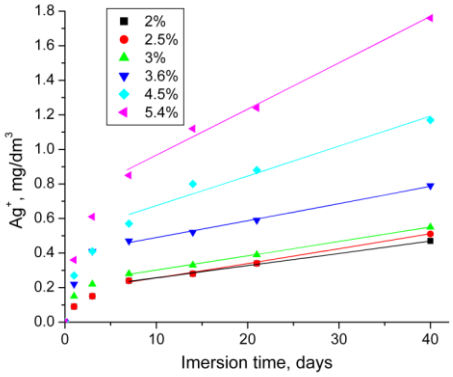


Fig. 5. 10. Silver release in time from organic matrix composites (OMCs)

Table. 5.3. Silver content (wt%) of the disk samples and the diameter of the zones of inhibition at 48 h

Tested strains	Silver content (%) and diameter of the zones of inhibition (mm)						
	2	2.5	3	3.6	4.5	5.4	0
Escherichia coli	10	12	12	12	14	15	0
Staphylococcus aureus	11	12	13	13	15	16	0
Staphylococcus spp.	0	8	10	10	12	14	0
Bacillus cereus	0	10	11	11	12	14	0
Candida albicans	10	11	12	12	15	15	0

These effects clearly releave that AgNPs can be efficiently used combined with multi-substituted HAP and a polymeric matrix, both with the role of carriers, in order to improve their efficiency against different pathogenic species. These composites can be considered as a promising antimicrobial material for coatings on orthopedic and dental implants, or for bone cements in surgical applications.

The rate of silver release from these composites in simulated body

fluid is decreasing in the first days, and becomes practically constant after 7 days. The antimicrobial effectiveness tests of the silver

containing composites, made against five different pathogenic species showed a clear inhibitory effect in all the microbial species tested.

This antimicrobial effect increases with the increase in silver content of the composites. Moreover, the medium remained sterile even after removing the silver containing composite disks, suggesting, that the antimicrobial effect of our compounds is of bactericidal nature.

Therefore, these composites have a strong activity against the 5 bacterial strains. On the other hand, multi substituted HAP with Zn (0.200%), Ag (0.250%) and Au (0.025 %) without silver nanoparticles did not show any antibacterial effect at these concentrations. These results suggest that our composites should be considered antibacterial materials and might be used in implants for dental and orthopedic surgical applications. The Ag^+ ions reacted with the five pathogens inactivating their metabolism, inhibiting their growth and finally determining the bactericidal activity of these composites containing HAP and AgNPs.

Future experiments are planned for *in vivo* evaluation that /HAP/ AgNPs /organic matrix nano composites might represent potential agents for coating of implants and thus avoiding or controlling bacterial infections - in orthopedic and dental surgery.

Recently we have showed that disks made form HAP nanoparticles, containing nitroxoline (5-nitro-8-hydroxyquinoline, NHQ) and Ag^+ ions lead to an increased antimicrobial activity against cultures of *Staphylococcus aureus*, as compared with the NHQ activity [A. Danistean, M. Gorea, A. Avram, S. Rapuntean, Gh. Tomoaia, A. Mocanu, **C. Garbo,** O. Horovitz, M. Tomoaia-Cotisel, Antimicrobial activity of ceramic disks loaded with silver ions and nitroxoline, *Studia Univ. Babes-Bolyai, Chemia*, **61** (3), Tom I, (2016) 275-283].

Within our recent interdisciplinary research about *in vitro* sensibilizing of micro-organisms against multi-functional nanostructures based on hydroxyapatite: HAP, hydroxyquinoline: HQ or nitro-hydroxyquinoline: NHQ, in presence of Zn^{2+} ions, we evidenced the remarkable biological antimicrobial effects on the following microbial strains: *Escherichia, Staphylococcus, Micrococcus, Bacillus, Candida şi Prototheaca:* [S. Rapuntean, A. Pop, V. Miclaus, **C. Garbo**, F. Chirila, Gh. Rapuntean, N. Fiţ, H. Farcău, M. Tomoaia-Cotisel, "Research concerning in vitro sensitivity of some microorganisms at hydroxyquinoline and cupric derivatives, deposited onto hydroxyapatite", Bulletin UASVM, Veterinary Medicine 72(2)/2016; pISSN 1843-5270; eISSN 1843-5378].

6. Mg, Zn, Sr, Si DOPED HYDROXYAPATITES NANOSTRUCTURES

Synthetic nano powders of multi-substituted hydroxyapatite, containing Mg, Zn, Sr and Si within hydroxyapatite (HAP) lattice, are prepared by a wet chemical method, without using surfactants or templates. The process parameters are set up to allow the simultaneous substitution of ions in the place of calcium as well as phosphate and OH^- groups. The four new hydroxyapatites were synthesized in premiere, namely: a complex HAP, HAP-1.5 wt% Mg-0.2%Zn-0.2%Si, HAPc; HAPc with 5%Sr, HAPc-5%Sr; and HAPc-10%Sr, including pure HAP, obtained in the same conditions (Table 6.1).

Table 6. 1. Composition of the prepared hydroxyapatites

No*	Sample	Composition (wt %)						Theoretical formula
		Ca	Mg	Zn	Sr	P	Si	
1,2	HAP	38.89	0	0	0	18.50	0	$Ca_{10}(PO_4)_6(OH)_2$
3,4	HAPc	37.71	1.5	0.2	0	18.47	0.2	$Ca_{9.36}Mg_{0.61}Zn_{0.03}(PO_4)_{5.93}(SiO_4)_{0.07}(OH)_{1.93}$
5,6	HAPc_Sr5%	34.34	1.5	0.2	5	17.97	0.2	$Ca_{8.76}Mg_{0.63}Zn_{0.03}Sr_{0.58}(PO_4)_{5.93}(SiO_4)_{0.07}(OH)_{1.93}$
7,8	HAPc_Sr10%	30.98	1.5	0.2	10	17.46	0.2	$Ca_{8.12}Mg_{0.65}Zn_{0.03}Sr_{1.20}(PO_4)_{5.93}(SiO_4)_{0.07}(OH)_{1.93}$

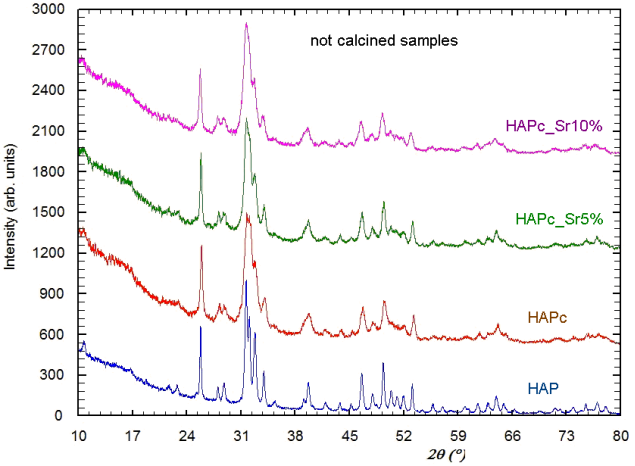
* Samples No 1, 3, 5, 7 are non calcined, while samples No 2, 4, 6, 8 are calcined samples (300°C, 1h)

The chemical, structural and morphological characterizations of the prepared powders are performed by XRD (Fig, 6.2), FTIR and Raman spectroscopy (Figs 6.3 and 6.4), TEM, HR-TEM, SEM (Fig. 6.11) and AFM as well as BET measurements (Fig. 6.22 and Tab. 6.6). The results show that, the ion substitution in the HAP lattice cause a distinct change in the shape and the size of nanoparticles or in the degree of crystallization of the produced pastes or lyophilized powders, non-calcined or calcined.

The ***degree of ion release*** of these nanopowders was determined in pure water and simulated body fluid. The multi-substituted hydroxyapatite (msHAP) with the highest Sr content showed the highest solubility with greater rate of ion release, compared with pure HAP powder, related to their enhanced biological activity.

The synthesized multi-substituted hydroxyapatites have the advantage of inheriting the *in vivo* effect of substituting elements, but also the structure and properties if pure HAP. As well they are biocompatible,

nontoxic, osseoconductive, non-immunogenetic, non- inflammatory and bioactive, having the ability to be integrated in the living tissue. Thus, the HAP biomaterials, multi-substituted or doped with different elements present chemical and structural similarities with bone minerals, and are promising candidates for bone substitutes, and for healing and remodeling the osteoporotic bone.



Fig, 6.2a. Comparison of XRD patterns for non calcined samples (1, 3, 5, 7)

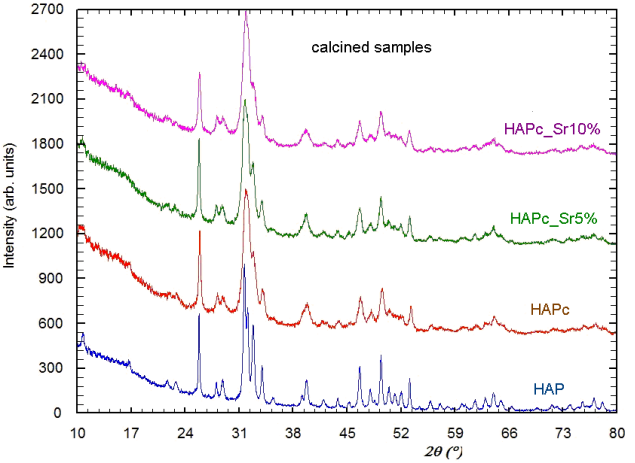


Fig. 6.2b. Comparison of XRD patterns for the calcined samples (2, 4, 6, 8)

The FTIR spectra are compared in Fig. 6.3a for the non calcined samples, and in Fig. 6.3b for the calcined ones. All the spectra are normalized to 1 for the highest absorption maximum.

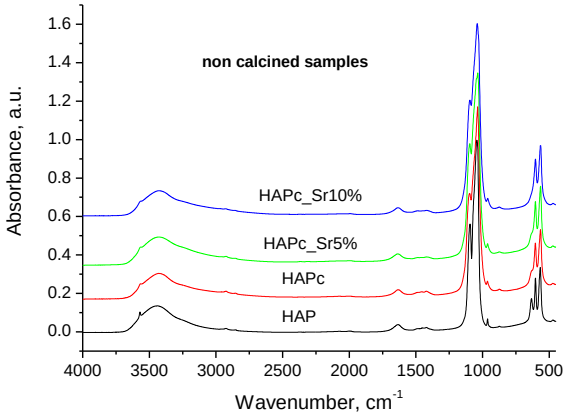


Fig. 6.3a. FTIR spectra of non calcined samples (1, 3, 5, 7); the spectra are normalized

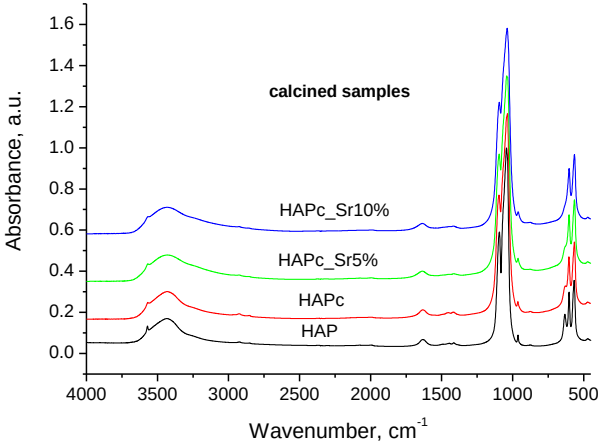
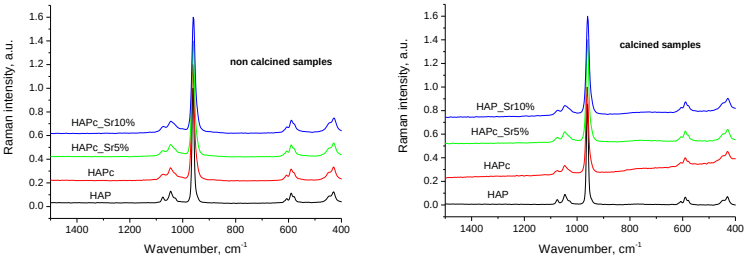


Fig. 6.3b. FTIR spectra of calcined samples (2, 4, 6, 8); the spectra are normalized

The spectra are similar, presenting all the absorption peaks for the PO_4 group in hydroxyapatites: asymmetric stretching ν_3 (the most intense band, doublet), symmetric stretching ν_1 (low intensity, since forbidden in IR), and the tow bending modes: asymmetric ν_4 (doublet) and symmetric ν_1. The characteristic peak for the structural OH groups in HAP is also present at 3430-3440 cm^{-1}, while the libration peak at about 630 cm^{-1} is visible only as a shoulder in most samples.

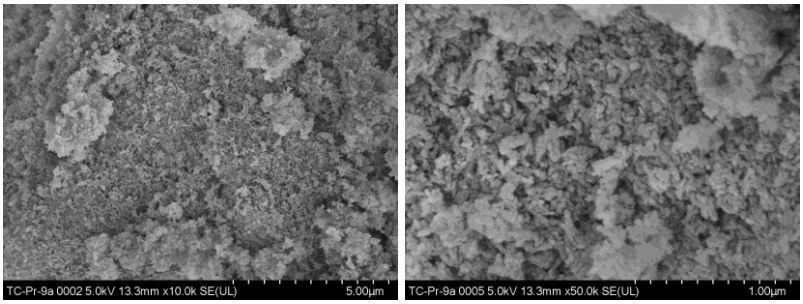
The characteristic vibrations for the PO_4 group appear all in the Raman spectra. The most intense is the symmetric stretching vibration ν_1, at 960-962 cm^{-1}, forbidden in IR.



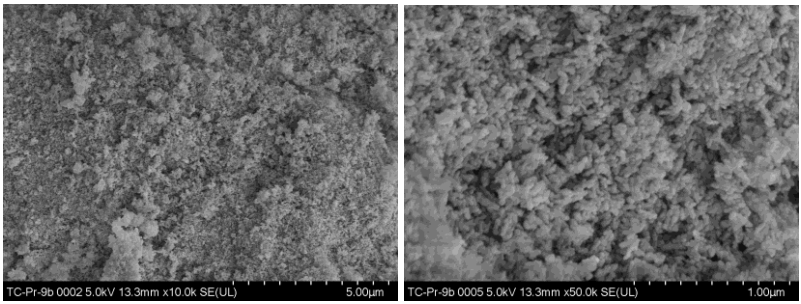
a. b.

Fig. 6. 4. Raman spectra of the (a) non calcined (1, 3, 5, 7), and (b) calcined (2, 4, 6, 8) samples; the spectra are normalized

TEM images allow us to see details on particles morphology. In the TEM images there are mostly oblong particles, with a length of 40-50 nm, and diameters of 20-30 nm. These oblong particles are made of uni-dimensional rows of mostly spherical domains



a. b.



c. d.

Fig. 6. 11. SEM images for HAP, non calcined sample 1 (a, b) and calcined sample 2 (c, d). the bars are 5 µm (a, c), respectively 1 µm (b, d).

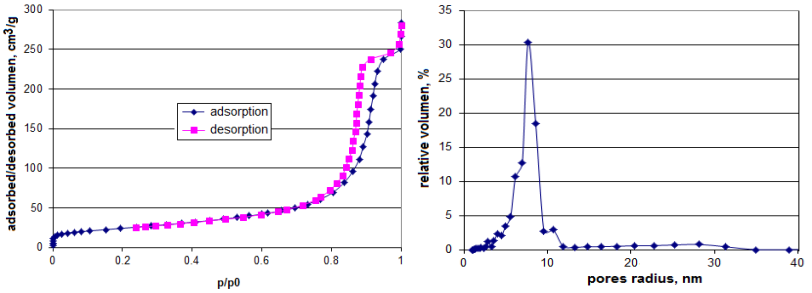
Some SEM images are given in Fig. 6.11. The SEM images show that the samples contain highly porous particles blocks, of micrometrical size. For higher magnification ratios the component nanoparticles can also be observed.

The results of BET analysis in terms of specific surface area, pores specific volume and most probable pore radius are presented in Table 6.6 A representative adsorption-desorption isotherms and pores size distributions for a calcined sample are given in Fig. 6.22.

All the samples present a rather high specific surface area, due both to the high degree of dispersion of the powders, and to their porosity. By calcination the specific surface area is diminished as an effect of nanoparticles sintering, while the pores specific volume is rather unchanged. The non-substituted HAP, both not calcined and calcined, presents a narrower distribution of pores sizes (about 8 nm dameter) then the substituted ones, where most pores have diameters between 10 and 25 nm.

Table 6.6. Some characteristics from BET analysis of HAP samples.

Sample	Specific surface area, m^2/g		Pores specific volume, cm^3/g		Most probable pore radius, nm	
	not calcined	calci-ned	not calcined	calci-ned	not calcined	Calci-ned
HAP	95.6	86.3	0.358	0.344	7.7	7.7
HAPc	135.1	107.4	0.376	0.383	11.3	9.5; 14.5
HAPc_5%Sr	106.1	99.9	0.229	0.237	20.2	14.5
HAPc_10%Sr	125.8	114.4	0.333	0.302	13.3	14.5



a. b.

Fig. 6. 22. Adsorption-desorption isotherms (a), and pores size distribution (b) for HAP calcined sample 2

The effect of substituting elements Sr, Mg, Zn, and Si on the structure and morphology of HAP was investigated, using various investigation techniques: XRD, FTIR and Raman spectroscopy, TEM, SEM and AFM imagistic, zeta potential and BET measurements, as well as the solubility study in different aqueous media: in ultrapure water and in SBF. Substituted hydroxyapatites release a larger amount of Ca and P than pure HAP, i.e. the presence of substituting elements increases the solubility of HAP. The amount of released P is higher than that corresponding to the stoichiometrical ratio P/Ca, so there is an incongruent dissolution. Release amounts of Mg and Sr are also larger than those corresponding to their content în the solid sample; magnesium seems to be released the easiest. The pH value of the medium presented some small variations between 6 and 7

7. BIOCOMPATIBILITY. Nanostructures of Mg, Zn, Sr, Si doped hydroxyapatites in osteoblasts cell cultures

The cellular response of osteoblasts in vitro to nanostructures made of hydroxyapatites doped with Mg (1.5 wt%), Zn (0.2 wt%), Sr (5 wt% and 10wt%), and Si (0.2wt%), self-assembled within 4 porous scaffolds made of stoichiometric HAP, HAP-Mg-Zn-Si (noted HAP complex: HAPc), HAPc-5%Sr and HAPc-10% Sr was assessed in culture medium for periods of 1, 3, 7, 21, 28 days. Viability tests, including adhesion and proliferation by using MTT, Alamar blue, and diacetyl fluorescein, as well as the cellular protein expression for osteoblasts markers, such as collagen-1, alkaline phosphatase, osteopontin, osteocalcin and osteonectin by utilizing the suitable cellular tests, demonstrated the benefits to early time points (<1

week) for bone formation through the incorporation of Mg, Zn and Si in porous hydroxyapatite lattice (HAP-Mg-Zn-Si) , and for HAP-Mg-Zn-Si-Sr, at both levels of Sr (5% and 10%), as compared to stoichiometric HAP. The nanostructures containing 5 wt% Sr or 10 wt% Sr supported significantly an enhancement of cellular response than all other groups HAP and HAPc at all time points, resulted from statistical analysis ($P< 0.05$). These results highlight the cellular response to Sr levels and suggest that a rather optimum response is obtained when HAP is substituted with 1.5%Mg, 0.2%Zn, 0.2%Si, and 10%Sr, through the effect of all elements used for doping HAP on the osteoblasts activity, and particularly at higher time points (> 1 week). Undoubtedly, besides chemical compositions of these biomaterials an important role is played by the surface structure and properties, such as surface roughness, of scaffolds on the interaction of biomaterials and cells at the interface of scaffolds with culture media. Through the recognized role of Sr on the bone resorbing cells, such as osteoclasts, another benefit can rationally be envisaged through the decreasing of bone resorption processes. In consequence, we suggest a potential application of these biomaterials in the treatment of osteoporosis, where a beneficial balance between the bone formation and the bone resorption might be achieved.

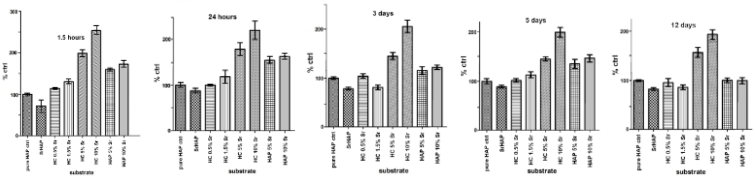
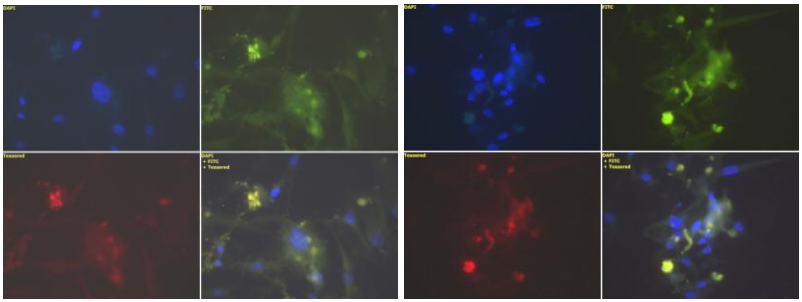


Fig. 7. 4. Graphical aspect of the viability test with Alamar blue evaluate at different time points, using scaffolds made of **HAP**: $Ca_{10}(PO_4)_6(OH)_2$ used as control; **SrHAP**: $Sr_{10}(PO_4)_6(OH)_2$; **HC**: complex HAP [HAP-Mg(1.5 wt%)-Zn(0.2 wt%)-Si(0.2 wt%)]; **HC**-0.5 wt% Sr, **HC**-1.5 wt% Sr, **HC**-5 wt% Sr, **HC**- 10 wt% Sr; HAP-5 wt% Sr; HAP-10 wt% Sr, developed within this scientific and technological research and teste don cell cultures.

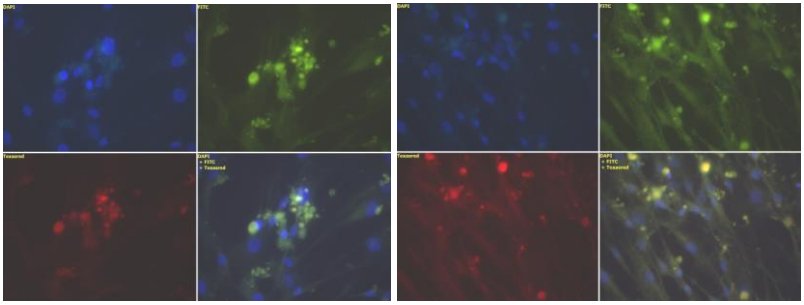
Viability tests Alamar Blue were applied to study the cell adhesion (in the first hours after seeding), and cell proliferation at different rime points, using as control cells grown on oure nano-HAP surfaces (Fig. 7.4). At 1.5 h after seeding the cells on the scaffolds surface, we find **HAPc:** HC (HAP-Mg-Zn-Si) doped with 5% Sr and 10% Sr to be the most favorable scaffolds for an enhanced cell adhesion, as also shown by the FDA coloring (diacetyl fluoresceine). The same situation is found also for other time

points, up to 12 days. Further, the biocompatibility and osteoinductive ability of different scaffolds made form HAPs with various Mg, Zn, Sr, Si contents on the behavior of osteoblasts was investigated, as regards their final differentiation to osteocytes



(a) (b)

Fig. 7.5. Immuno-staining of osteoblasts for collagen, in green with FITC, osteocalcin, red with Texas red, cell nuclei, in blue, stained with DAPI. Images have been made at 7 days of osteoblasts culture on scaffolds made from pure HAP (a) and complex HAP: HC (b): HAP-Mg(1.5%)-Zn(0.2%)-Si(0.2%)-Sr(1.5%).



(a) (b)

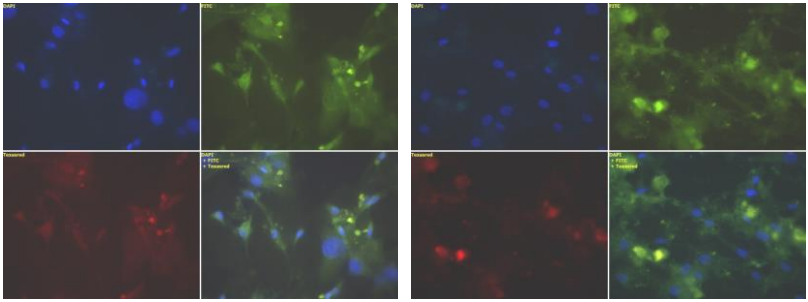
Fig. 7. 6. Immuno-staining of osteoblasts for collagen, in green with FITC, osteocalcine, red wirh Texas red, cell nuclei, blue stained with DAPI. Images were taken after 7 days of osteoblasts culture on scaffolds, made (a) from HAP-Mg(1.5%)-Zn(0.2%)-Si(0.2%)-Sr(5%), and (b) from HAP-Mg(1.5%)-Zn(0.2%)-Si(0.2%)-Sr(10%).

The initiation of the mineralizationprocess after 7 days of culture was investigated by immunocytochemical staining for the expression of bone protein, such as osteocalcine and osteopontine and for the *de novo*

synthesis of collagen. All these proteins are secreted by osteoblasts and are components of the extracellular bone matrix. Osteocalcine is evidenced by red color (texas red), collagen-1A in green (FITC), and cell nuclei in blue (DAPI).

Following the interaction of HAP and the osteoblasts, a matrix was developed (Fig.7.5a), containing collagen (green), which can be observed both as fibrils and as globular agglutinates, and osteocalcine (red). On the contrary, HC scaffolds with 1.5% Sr (Fig. 7. 5b) present a less organized matrix, but also sustain the proliferation (high density of cell nuclei, colored in blue).

Scaffolds made from HAP-Mg(1.5%)-Zn(0.2%)-Si(0.2%)-Sr(5%) cell proliferation even after 7 days (Fig. 7. 6a), while developing a bone matrix with more defined crystals and collagen fibrils. A similar aspect was observed on scaffolds from HC-10% Sr [HAP-Mg(1.5%)-Zn(0.2%)-Si(0.2%)-Sr(10%), images given in Fig. 7. 6b, were an enhanced organization of collagen fibrils is observed. A strong expression for osteocalcine and collagen 1a was also remarked.



(a) (b)

Fig. 7. 7. Immuno-staining of oosteoblasts for collagen, in green with FITC, osteocalcine, red wirh Texas red, cell nuclei, blue stained with DAPI. Images were taken after 7 days of osteoblasts culture on scaffolds, made (a) from HAP-Sr(5%), and (b) from HAP-Sr(10%).

Scaffolds made form HAP with 5% Sr and HAP-10% Sr proved to be the most favorable for cell proliferation. A stronger expression for osteocalcine was observed on HAP-5% Sr scaffold (Fig. 7.7a), and a more advanced organization of collagen 1A fibers, on HAP-10% Sr support (Fig. 7. 7b).

The obtained results prove the stimulating role of the developed biomaterials, based on doped (multi-substituted) hydroxyapatites with Mg,

Zn, Sr, Si (HAP-Mg-Zn-Sr-Si) on cell viability, proliferation and differentiation in culture medium.

This new generation of bioactive materials, HAP-Mg-Zn-Sr-Si, is intensively studied as regards the induction of a rapid osseointegration with the host bone, by promoting the cell proliferation and differentiation, as well as by activation of the specific cell signaling paths and improving gene expression. The incorporation of certain elements, such as strontium, zinc, magnesium, and silicon in bone grafts presents a particular interest, dur to their role as co-factors in metabolic processes of the bone and cartilages, and also for the ionic dissolution products, which can stimulate the cell proliferation and the production of growth factors and extracellular matrix.

The obtained results suggest that these biomaterials, HAP-Mg-Zn-Sr-Si, have potential applications in the bone tissue repair and in the treatment of osteoporotic bone fractures.

8. NANOSTRUCTURES: HYDROXYAPATITE, AgNP IN POLYMER MATRIX. New bone cement of biomedical interest.

Although total joint replacement surgery has become common in recent years, problems due to bacterial infection remain a significant complication following this procedure. The objective of this study was to obtain a self-cured bone cement (Table 8.1), based on hydroxyapatite (HAP) with silver nanoparticles (AgNPs) noted Hap-Ag, (characterized by XRD, Fig. 8.3), and ZrO_2 embedded in a polymer matrix based on 2,2-bis[4-(2-hydroxy-3-methacryloyloxypropoxy)-phenyl]propane-triethyleneglycol dimethacrylate. [G. Furtos, M. Tomoaia-Cotisel, **C. Garbo**, M. Şenilă, N. Jumate, I. Vida-Simiti, C. Prejmerean, New composite bone cement based on hydroxyapatite and nanosilver, *Particulate Science and Technology,* **31** (4), 392-398 (2013)]. The interaction of the inorganic powder with water depend on the type, content and size of the filling, as well as on the characteristics of the polymer matrix: polarity (hydrophilic or hydrophobic) and degree of conversion. The new nanostructured composite biomaterials obtained were tested for: in vitro Ag^+ ions release, which increased in time and was dependent on the silver content in the cement. The highest silver release was observed for the cement with 1.26 wt% silver.

These nanostructured biomaterials were also tested for mechanical properties: compressive strength (CS), compressive modulus (CM), and also for radiopacity.

Compressive strength (CS) was determined in a universal testing machine (Lloyd LR5Kplus, UK) at a loading rate of 0.75mm/min until

fracture. The load deflection curves were recorded with computer software (Nexygen; Lloyd Instruments). The CS value in MPa was calculated by the following equation 3:

$$CS = \frac{F}{\pi \cdot r^2}, \qquad (3)$$

where F is the applied load (N), and r is the radius of the cylindrical sample measured before testing (2mm). CM (GPa) value was determined from the slope in the elastic portion of the stress-strain curve (dσ-dε).

The results revealed that the CS for bone cement was between 133.37 and 146.70 MPa, and CM was between 1.68 and 1.82 GPa ($p>0.05$). The highest CS values were obtained for C1 and C3 cements, and this behavior could be explained by the higher degree of cross binding in the polymer matrix. A slight increase of CM was observed for the samples with a 1.5/1 powder/liquid ratio. Adding AgNP and ZrO_2 increased the radiopacity of the experimented bone cement.

Table 8. 1. Composition of bone cements

Code cement	Components (wt%.)	P/L*
C0	Hap (50%), Monomers (50%)	1/1
C1	Hap_Ag (50%), Monomers (50%)	1/1
C2	Hap_Ag (60%), Monomers (40%)	1.5/1
C3	Hap_Ag (25%), ZrO_2 (25%), Monomers (50%)	1/1
C4	Hap_Ag (35%), ZrO_2 (25%), Monomers (40%)	1.5/1

*P = powder; L = monomers liquid

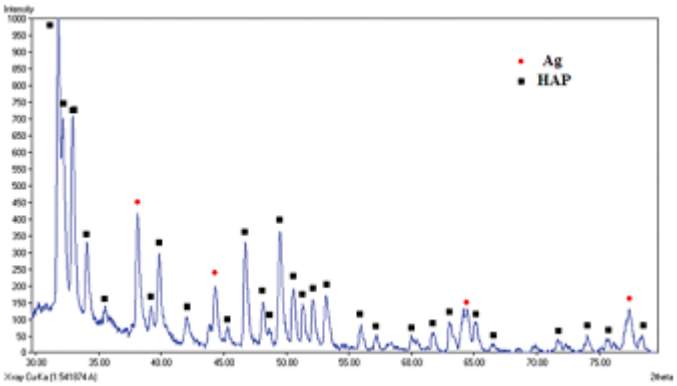


Fig. 8.3. Powder X-ray diffraction patterns of Hap_Ag.

An increase of radiopacity values from 0.38 mm Al for C0 cement to 3.91mm Al for C4 cement was observed. The results show a high difference between radiopacity of cements with HAP, HAP_Ag powder and cements with HAP_Ag and ZrO_2 powder.

In order to improve the lifetime of cemented implants, and, consequently, the patient care, it is of vital importance to improve de mechanical properties, the radiopacity and the biocompatibility of the materials, or to obtain the formation of new bone at the cement-bone interface.

Adding AgNP in these biomaterials aimed to assure an antimicrobial activity. The silver nanoparticles present efficient antimicrobial properties, due to their extremely large surface area, which assures a good contact with the microorganisms. Using AgNP as filler in composite materials will increase the dispersion of silver particles at the bone cement surface and at the bone-cement interface.

Increasing of the P/L ratio (Table 8.1) and adding of AgNP and ZrO_2 powders helped to improve the cements radiopacity in the following order: 0.38 mmAl (C0)< 0.88 mmAl (C1)<1.24mmAl (C2)<3.47mmAl (C3)<3.91mmAl (C4).

The obtained results recommend these biomaterials for potential clinical applications.

9. GENERAL CONCLUSIONS

The ***PhD Thesis*** presents strategies for safe production of nanostructures resulting in realization of ***innovative biomaterials,*** designed to ***stimulate cellular responses*** at molecular level.

The obtained biomaterials were characterized by adequate physical, chemical, and biological methods: XRD, FTIR and Raman spectroscopies, TEM, SEM, AFM, BET and zeta potential measurements, and by biological and biophysical tests, proving osteoblasts adhesion, proliferation, and differentiation *in vitro*.

The obtained results show that modifications in the composition and structure of hydroxyapatite can accelerate the bone formation and remodeling, with potential biological and biomedical applications.

In **chapter 1**, ***seven series of nanostructures*** **(**biomaterials) were prepared**,** based on hydroxyapatites, with controlled properties: different crystallinity degree and various nanoparticles sizes and shapes, calcined or non-calcined. These characteristics assure a good biological activity, for potential biomedical utilizations.

The research objective of **chapter 2** was to investigate the effects of silicate substitution within hydroxyapatite (HAP) lattice on its chemical and physical properties and in vitro osteoblast biological response. A wet chemical method was used to synthesize ***four*** silicate substituted HAP (Si-HAP) powders with 0.47 wt%, 2.34 wt%, 4.67 wt% and 9.34 wt% Si, freeze dried and then calcined at 650 ^{o}C for 1h.

Biomaterials were physically and chemically characterized, by XRD, FTIR and Raman spectroscopy as well as by TEM, SEM and AFM imaging, and investigated regarding their influence on cellular adhesion, proliferation, and protein expression, in human osteoblasts culture, using viability tests, MTT and Alamar blue, and immunocytochemical staining of osteoblasts, considering their potential use in bone repair and regeneration.

The scaffolds of Si-HAP nanostructures have a better enhanced activity on osteoblasts than pure HAP. Clearly, the scaffolds made of HAP-Si(0.47%) and HAP-Si(2.34%) appear to have the best activity from the four nano materials Si-HAP enriched in Si content for proliferation of osteoblasts. These results suggest that Si-HAP biomaterials play an important role in regulating the proliferation and osteogenic differentiation of osteoblasts into osteocytes, as well as in the bone formation, having potential applications in defect bone repair.

In **chapter 3** the synthesis of ***five series*** of new porous nano-hydroxyapatites (HAP), substituted with different amounts of Zn (HAP-Zn), from 0.2 to 10 wt%, in presence of a surfactant, L-asparagine, to control the pores size distribution and the morphology of the nanopowders, obtained by a co-precipitation process. Thus a new approach was developed for preparing HAP-Zn nanopowders, with improved nucleation and crystallization ability. XRD measurements indicated HAP to be the single phase present and evidenced the nanostructured nature of these biomaterials. FTIR spectra also confirmed the presence of HAP structure. TEM, SEM, and AFM images showed the morphology of the HAP-Zn nanostructures. BET analysis allowed us to characterize the specific surface area and samples porosity. The new nanopowders are designed to be used in orthopedic surgery, particularly for the treatment of osteoporosis and as bone substitutes, and in dentistry for enamel remineralization.

In **chapter 4**, the preparation of ***four*** new strontium substituted hydroxyapatites, HAP-Sr, with high crystallinity, is described, using a new wet chemical methodology, assisted by templates (ethylenediamine and o-toluidine). Synthesized hydroxyapatites correspond to the formula $Ca_{10-x}Sr_x(PO_4)_6(OH)_2$ with x = 0 (pure hydroxyapatite, HAP), 0.59, 1.21 and 1.87 (HAP-Sr) as well as 10, corresponding to Ca totally substituted by Sr (SrHAP). They were characterized by XRD, FTIR and Raman

spectroscopy, as well as by TEM and AFM. These new HAP-Sr nanomaterials have potential applications in biomedical engineering, especially in orthopedic, dental and maxillofacial field.

In **chapter 5,** nano hydroxyapatite doped with Ag (0.25 wt%), Au (0.025 wt%), and Zn (0.2 wt%), (HAP-Ag-Au-Zn), has been obtained in premiere by an innovative wet chemical approach, coupled with a reduction process. Nano HAP-Ag-Au-Zn was mixed with silver nanoparticles (AgNP), previously synthesized, obtaining a new material: HAP-Ag-Au-Zn with AgNP, named HAP-Ag. Finally, organic matrix composites were obtained, with HAP and/or HAP-Ag as filler, and a mixture of monomers (bis-GMA, 60% and TEG-DMA, 40%), which were polymerized at various compositions in silver content up to 5.4 wt%. These ***eight series*** of new composites present significant antimicrobial properties. Their inhibitory effect was observed against 5 tested microbial species: *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus spp*., *Bacillus cereus*, and *Candida albicans*.

Within recent interdisciplinary research, we showed that hydroxyapatites act as carrier for AgNP or various complexes of silver with different biomolecules, or zinc and different biomolecules, with important antimicrobial effects against pathogens, such as *Escherichia coli, Staphylococcus, Micrococcus, Bacillus, Candida,* and *Prototheca.*

The obtained results showed that these composite can be considered as a promising antimicrobial material, for coatings on orthopedic or dental implants, or for bone cements in surgical applications, or in the treatment of various infections in animals.

Chapter 6 presents multi-substituted hydroxyapatite, containing Mg, Zn, Sr and Si within hydroxyapatite (HAP) lattice, prepared by a wet chemical method, which allows the simultaneous substitution of ions in the place of calcium as well as phosphate and OH^- groups. The ***four new*** hydroxyapatites were synthesized in premiere, namely: a complex HAP, HAP-1.5 wt% Mg-0.2%Zn-0.2%Si, HAPc; HAPc with 5%Sr, HAPc-5%Sr; and HAPc-10%Sr, including pure HAP, obtained in the same conditions.

The effect of substituting elements Sr, Mg, Zn, and Si on the structure and morphology of HAP was investigated, using various investigation techniques: XRD, FTIR and Raman spectroscopy, TEM, SEM and AFM imagistic, zeta potential and BET measurements, as well as the solubility study in different aqueous media: in ultrapure water and in SBF.

Substituted hydroxyapatites release a larger amount of Ca and P than pure HAP, i.e. the presence of substituting elements increases the solubility of HAP. The amount of released P is higher than that corresponding to the stoichiometric ratio P/Ca, so there is an incongruent

dissolution. Release amounts of Mg and Sr are also larger than those corresponding to their content in the solid sample; magnesium seems to be released the easiest. The pH value of the medium presented some small variations between 6 and 7.

Chapter 7 presents the cellular response of osteoblasts in vitro to nanostructures made of hydroxyapatites doped with Mg (1.5 wt%), Zn (0.2 wt%), Sr (5 wt% and 10wt%), and Si (0.2wt%) (synthesized in Chapter 6), self-assembled within ***4 porous scaffolds*** made of stoichiometric HAP, HAP-Mg-Zn-Si (noted HAP complex: HAPc), HAPc-5%Sr and HAPc-10% Sr, which was assessed in culture medium for periods of 1, 3, 7, 21, 28 days. Viability tests, including adhesion and proliferation by using MTT, Alamar blue, and diacetyl fluorescein, as well as the cellular protein expression for osteoblasts markers, such as collagen-1, alkaline phosphatase, osteopontin, osteocalcin and osteonectin by utilizing the suitable cellular tests were applied. Their results highlight the cellular response to Sr levels and suggest that a rather optimum response is obtained when HAP is substituted with 1.5%Mg, 0.2%Zn, 0.2%Si, and 10%Sr, through the effect of all elements used for doping HAP on the osteoblasts activity. The obtained results suggest that these biomaterials, HAP-Mg-Zn-Sr-Si, have potential applications in the bone tissue repair and in the treatment of osteoporotic bone fractures.

Chapter 8 presents nanostructures made from hydroxyapatite, silver nanoparticles, AgNP, and ZrO_2, embedded in a polymer matrix, obtaining ***four new series*** of bone cements of biomedical interest. The obtained composite nanostructured biomaterials were tested for Ag^+ ions release, and for mechanical properties: compressive strength (CS), compressive modulus (CM), and also for radiopacity. The obtained results recommend these biomaterials, with enhanced antimicrobial effects, for potential clinical applications.

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11. Published articles: *UEFISCDI awarded works*

Own scientific works (original research); two UEFISCDI awarded works
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1. A. Mocanu, R.D. Pasca, Gh. Tomoaia, **C. Garbo**, P. T. Frangopol, O. Horovitz and M. Tomoaia-Cotisel, "New procedure to synthesize silver nanoparticles and their interaction with local anesthetics", *Int. J. Nanomedicine*, **8,** (2013), pp. 3867-3874
2015 Impact Factor*:* **4.320** *(5 year impact **5.034**). **UEFISCDI awarded work***

2. G. Furtos, M. Tomoaia-Cotisel, **C. Garbo**, M. Şenilă, N. Jumate, I. Vida-Simiti, C. Prejmerean, "New composite bone cement based on hydroxyapatite and nanosilver", *Particulate Science and Technology*, **31** (4), (2013), pp. 392-398
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3. A. Mocanu, G. Furtos, S. Rapuntean, O. Horovitz, C. Flore, **C. Garbo**, A. Danisteanu, Gh. Rapuntean, C. Prejmerean, M. Tomoaia-Cotisel, „Synthesis; characterization and antimicrobial effects of composites based on multi-substituted hydroxyapatite and silver nanoparticles", *Applied Surface Science*, **298**, (2014), pp. 225–235
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8. S. Rapuntean, A. Pop, V. Miclaus, **C. Garbo**, F. Chirila, Gh. Rapuntean, N. Fiț, H. Farcău, M. Tomoaia-Cotisel, "Research concerning in vitro sensitivity of some microorganisms at hydroxyquinoline and cupric derivatives, deposited onto hydroxyapatite", Bulletin UASVM, Veterinary Medicine 72(2)/2016; pISSN 1843-5270; eISSN 1843-5378.

12. Communications at Conferences and Symposia: *Awarded Poster*

1. Cristina Prejmerean, Ioan Petean, Aurora Mocanu, Gabriel Furtos, **Corina Garbo**, Moldovan Marioara, Maria Tomoaia-Cotisel, "*Surface structure of three bioadhesive systems with potential applications in dentistry. AFM investigations*", 1st International Conference on Biological and Biomimetic Adhesives, COST Action TD 0906, School of Dentistry, University of Lisbon, Portugal, **9-11 May, 2012.**

2. Cristina Prejmerean, Ioan Petean, Aurora Mocanu, Gabriel Furtos, **Corina Garbo**, Moldovan Marioara, Maria Tomoaia-Cotisel, "*Surface morphology of some bioadhesives with potential applications in medicine. AFM investigation*", The 32nd National Conference of Chimistry, Caciulata, **3-5 October, 2012.**
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3. Valer Almășan, Mihaela Lazăr, Maria Tomoaia-Cotisel, Aurora Mocanu, **Corina Garbo**, Gheorghe Tomoaia, "*Modified nanostructured hydroxyapatite with medical applications*", The 8th International Conference on Materials Science & Engineering – BRAMAT 2013, Brașov, **28 February – 2 March, 2013.**
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4. Valer Almasan, Mihaela D. Lazar, Gheorghe Tomoaia, Aurora Mocanu, **Corina Garbo**, Laura Ciupeiu, Camelia Ciobotariu, Adriana Andonie, Cristina Spelmezan, Liviu Dorel Bobos and Maria Tomoaia-Cotisel, "*Modified nanostructured hydroxyapatite with biological and biomedical applications*", COST Action TD0906 "WG3 & WG4 Scientific Workshop Biological Adhesives: from Biology to Biomimetics", Cluj-Napoca, **9-11 April, 2013**

5. Cristina Prejmerean, **Corina-Lacramioara Garbo**, Hodisan Ioana-Maria, Ancuta Danisteanu, Ioan Petean, Aurora Mocanu, Gabriel Furtos, Maria Tomoaia-Cotisel, "*Surface characterization of some bioadhesives with potential applications in medicine. AFM investigation*", The 11th Conference on Colloid and Surface Chemistry – 11 CCSC, Iasi, **9-11 May, 2013.**
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6. Gheorghe Tomoaia, Olga Soritau, Aurora Mocanu, **Corina Garbo**, Maria Tomoaia-Cotisel, "*Scaffolds made of nano hydroxyapatite, collagen and chitosan*

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7. **Corina Garbo**, Ossi Horovitz, Aurora Mocanu, Valer Almasan, Gheorghe Borodi, Ioan Bratu, Maria Tomoaia-Cotisel, "*Effects of maturation conditions on the structure of hydroxyapatite nanopowder*", The 5th Conference on Advanced Spectroscopies on Biomedical and Nanostructured Systems, Cluj-Napoca, **7-10 September, 2014.**

8. **Corina Garbo**, Aurora Mocanu, Valer Almasan, Balint Reka, Gheorghe Borodi, Ioan Bratu, Ossi Horovitz , Maria Tomoaia-Cotisel,"*Effects of preparation conditions and the presence of surfactant on the hydroxyapatite nanopowders",* PIM 2015, Cluj-Napoca, **23 - 25 September, 2015.**
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9. Roxana-Diana Pasca, Gheorghe Tomoaia, Aurora Mocanu, Ioan Petean, **Corina Garbo**, Alexandra Gertrud Paltinean, Maria Tomoaia-Cotisel, *"The interaction between collagen, hydroxyapatite and APTES. Langmuir-Blodgett and AFM study"*, ICPAM 11, 11th International Conference on Physics of Advanced Materials, Cluj-Napoca, **8-14 September, 2016;** Poster premiat.
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10. Ancuta Danistean, **Corina Garbo**, Reka Balint, Gheorghe Tomoaia, Aurora Mocanu, Sorin Rapuntean, Ossi Horovitz, Maria Tomoaia-Cotisel, "*Bioceramics comprising silver nanoparticles, as a new generation of antimicrobials*", ICPAM 11, 11th International Conference on Physics of Advanced Materials, Cluj-Napoca, **8-14 September, 2016.**
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13. Participation in contractual research

She is a member in the research team of projects within the National Plan 2: DONTAS no. 171 and INOVAMAT no. 241, as well as in the international project NANOFOROSTEO no. 4-005, within the European Platform of Nanomedicine, realized in the Center of Physical Chemistry, Faculty of Chemistry and Chemical Engineering, UBB, Director, Prof. Univ. Dr. Maria Tomoaia-Cotisel.

14. Member in Chemical Societies and Research Centers

Member in the Chemical Society and in the Center of Physical Chemistry, Faculty of Chemistry and Chemical Engineering, Babes-Bolyai University of Cluj-Napoca.