UNIVERSITY OF MEDICINE AND PHARMACY
OF CRAIOVA
DOCTORAL SCHOOL

PhD THESIS SUMMARY

CHEMICAL SYNTHESIS AND CHARACTERIZATION OF SOME HYDROXYAPATITE COMPOSITES

SCIENTIFIC ADVISOR:
PROF. UNIV. DR. JOHNY NEAMȚU

PhD STUDENT:
BALOSACHE (NICOLAESCU) OANA ELENA

CRAIOVA
2014

CONTENTS

ABBREVIATIONS
INTRODUCTION
CURRENT STATE OF KNOWLEDGE
CHAPTER 1. HYDROXYAPATITE - AN ESSENTIAL COMPONENT OF BONE
1.1. Macroscopic structure of the bone
1.2. Microscopic structure of bone (bone microstructure and mesostructure)
1.3. Nanostructure of bone
1.4. Histogenesis of bone
1.5. Bioceramic based on calcium phosphates
1.6. Hydroxyapatite properties
1.7. Synthesis methods
1.7.1. Wet precipitation method
1.7.2. Hydrothermal synthesis
1.7.3. Sol – gel synthesis
1.7.4. Multiple emulsion method
1.7.5. Biomimetic synthesis
1.7.6. Electrodeposition

CHAPTER 2. BISPHOSPHONATES IN CURRENT THERAPEUTIC PRACTICE
2.1. Bisphosphonates classification
2.2. Structure-activity relationship
2.3. Pharmacological infornations
2.3.1. Therapeutic indications
2.3.2. The mechanism of action of bisphosphonates
2.3.3. Adverse effects of systemic treatment with bisphosphonates

ORIGINAL CONTRIBUTIONS
CHAPTER 3. PHYSICO-CHEMICAL CHARACTERIZATION OF SODIUM ALENDRONATE TRIHYDRATE
3.1. Alendronate solubility study
3.1.1. Materials and Methods
3.1.2. Results and Discussion
3.2. Dissociation constants calculation by potentiometric titration
3.2.1. Materials and Methods
3.2.2. Results and Discussion
3.3. Alendronate bioavailability

CHAPTER 4. SYNTHESIS AND CHARACTERIZATION OF HYDROXYAPATITE-ALENDRONATE COMPOSITES
4.1. Materials and Methods
4.1.1. Synthesis of hydroxyapatite by wet precipitation method
4.1.2. Synthesis of hydroxyapatite-alendronat composites
4.1.3. FT-IR characterization
4.1.4. X-ray diffraction characterization
4.1.5. Determination of particle size
4.1.6. Determination of alendronate concentration and incorporation efficiency by HPLC
4.2. Results and Discussion
4.2.1. Synthesis
4.2.2. FT-IR characterization

4.2.3. X-ray diffraction characterization
4.2.4. Determination of particle size
4.2.5. Determination of alendronate concentration and incorporation efficiency by HPLC
4.3. HA-AL synthesis optimization
4.4. Synthesis modeling using the experimental design- MODDE
4.4.1. Method
4.4.2. Results and Discussion

CHAPTER 5. HA-AL 20 mM COATING ON Ti SURFACES AND ITS CHARACTERIZATION
5.1. HA-AL 20 mM coating on Ti surfaces by pulsed laser evaporation (MAPLE)
5.1.1. Materials and Methods
5.1.2. Results and Discussion
5.2. HA-AL 20 mM characterization on Ti surfaces by AFM
5.2.1. Materials and Methods
5.2.2. Results and Discussion

CHAPTER 6. BIOCOMPATIBILITY ANALYSIS OF SYNTHESIZED BIOMATERIALS
6.1. Materials and Methods
6.2. Results and Discussion
6.2.1. Human bone cell proliferation in the presence of HA, alendronate and HA-AL
6.2.2. Human mesenchymal stem cell proliferation on HA-AL films

CHAPTER 7. IN VITRO RELEASE STUDIES OF ALENDRONATE FROM HA-AL COMPOSITES
7.1. In vitro release studies of alendronate from HA-AL 20 mM tablets
7.1.1. Materials and Methods
7.1.2. Results and Discussion
7.2. In vitro dissolution studies of AL after its chemisorption on HA coated metallic surface by MAPLE
7.2.1. Materials and Methods
7.2.2. Results and Discussion
7.3. In vitro dissolution studies of AL from HA-coated on metallic surface
7.3.1. Materials and Methods
7.3.2. Results and Discussion

GENERAL CONCLUSIONS. ORIGINAL CONTRIBUTIONS. RESEARCH PERSPECTIVES

REFERENCES

Keywords: *hydroxyapatite-alendronate composites; physico-chemical characterisation; implantable structures; release and biocompatibility studies;*

ABREVIERI

HA – hydroxyapatite
AL – alendronate
BF – bisphosphonates
SBF –Simulated Body Fluid
HA-AL –hydroxyapatite-alendronate composite
d-HA – calcium-deficient hydroxyapatite
FT-IR – Fourier transform infrared Spectrometry
HPLC – High performance liquid chromatography
DAD – Diode array detector
XRD – X-ray diffraction
AFM – Atomic force microscopy
PLD - Pulsed laser deposition
NCP – uncollagenic protein
MAPLE - Pulsed laser evaporation
SD - standard deviation
SRD – relative standard deviation
OB – osteoblastic cells
DLS –Dynamic light scattering
MCS – mesenchymal stem cells
LOD – detection limit
LOQ –quantification limit

INTRODUCTION

GENERAL CONSIDERATIONS

Recently, special attention is paid among researchers in order to obtain biomaterials used for reconstruction of bone tissue. Of these biomaterials, hydroxylapatite (HA) has a number of special properties, such as biocompatibility and bioactivity, which is commonly used in the preparation of bone grafts and coating of the metal components used in orthopedic prostheses.

Even if HA is available as a synthetic material for over 15 years and it is used in ceramic implants for over a decade, new solutions are seeking to incorporate into its structure certain anionic or cationic substituents which will bring closer the material to the composition of biological apatite.

Since the viability of the implant depends on processes occurring at the bone-implant interface, physico-chemical optimization of implants surface used in orthopedic surgery is essential to achieve a consistent and rapid bone integration[1].

There is a great interest among specialized physicists, biologists and physicians in developing biomimetic surfaces formed of calcium phosphate and proteins that would improve cell adhesion and thus would reduce bone integration time.[2] To prevent periprosthetic bone loss, it may be implemented antiresorptive drug therapy, achieved by including bisphosphonates that inhibit osteoclast activity[3]. Bisphosphonates are used in many diseases, such as Paget's disease of bone, osteoporosis, hypercalcemia[4].

Although the main effect of bisphosphonates is the inhibition of osteoclast bone resorption, there are studies showing a positive effect on osteoblasts too. Thus, numerous studies show a differentiated increase of osteoblast progenitor cells with positive influence on their proliferation and maturation. At the same time, these studies show that bisphosphonates prevent apoptosis of osteoblasts[5].

PROPOSED OBJECTIVES

In the attempt to avoid the problems generated by the prosthesis (bone loss) and possible side effects that may occur in long-term treatment with bisphosphonates, a commonly solution studied in recent years is local release of the bisphosphonate.

Thus it can be given a higher dose in the region of interest with effects on both the decrease in the loss of periprosthetic bone volume, but mostly with positive effects on reducing the time of osseointegration and accelerating the fixation of the prosthesis component. On the other hand, this leads to a stable attachment in the case of osteoporotic bone.

Implants with local bisphosphonates delivery were tested in various preclinical animal studies (rat, rabbit, dog) and the results were encouraging with regard to the viability of the implant.

The high affinity of bisphosphonates for the calcium ion and the advantages of the use of hydroxyapatite (HA) as the coating of prosthetic implants has led to:

- searching for new solutions to include drugs on prosthetic surface;

- the need to synthesize a hydroxiapatite-bisphosphonate compound, which might cover the implant;

Thus, in this study we aimed to obtain implantable structures by coating titanium metal substrates with alendronate-hydroxyapatite (HA-AL) composites.

Alendronate was chosen for this study from the class of bisphosphonates, its molecule contain a nitrogen atom (structurally similar to risedronate, and zoledronate), which gives it a structural conformation which potentiates its therapeutic effects.

The thesis is divided into two parts: a theoretical part (chapters 1-2) containing informations described in the scientific literature (the current state of knowledge) and an experimental part, divided into five chapters (chapters 3-7) and presenting experimental results.

CURRENT STATE OF KNOWLEDGE

CHAPTER 1. HYDROXYAPATITE - AN ESSENTIAL COMPONENT OF BONE

Chapter 1 contains a bibliographic study of literature describing the properties of natural hydroxyapatite as an essential component of bone and comparing the main methods for obtaining synthetic hydroxipatite.

The wet precipitation method (also called chemical precipitation) selected by us for this study, is the most popular technique used for the synthesis of hydroxyapatite. The absence of organic solvents and low production costs make this method to be intensively studied in the literature in the attempt to optimize the synthesis conditions.

CHAPTER 2. BISPHOSPHONATES IN CURRENT THERAPEUTIC PRACTICE

Chapter 2 deals with the use of bisphosphonates in the current therapeutic practice. Are specified concepts as bisphosphonates structure-activity relationship, mechanism of action, uses and adverse effects of systemic treatment with bisphosphonates.

The data related to structure-activity correlation provides several examples where small differences in the compounds structure of this class of drugs lead to surprising changes in bone affinity.

Therefore, alendronate was chosen for our study, since its spatial configuration (N-bisphosphonate) and nitrogen orientation into the structure is playing an important role in the affinity of the coordination to calcium atoms from hydroxyapatite, and implicitly it can be assumed that the affinity of binding to synthetic hydroxyapatite estimate the probability of its attachment to the bone.

CHAPTER 3. PHYSICO-CHEMICAL CHARACTERIZATION OF SODIUM ALENDRONATE TRIHYDRATE

Chapter 3 is a brief survey which characterized the alendronate used in the synthesis. It was thus determined its solubility and its dissociation constants were calculated by potentiometric titration.

CHAPTER 4. SYNTHESIS AND CHARACTERIZATION OF HYDROXYAPATITE-ALENDRONATE COMPOSITES

Chapter 4 covers the synthesis and characterization of hydroxyapatite-alendronate composites. The synthesis method allowed us to vary the parameters (synthesis temperature, pH, rate of addition of the reactants, rate of stirring of the reaction mixture) which may affect drastically the morphology, structure and crystal size of hydroxyapatite.

The method of synthesis of hydroxyapatite and HA-AL compounds by wet precipitation presents a series of advantages like: simplicity in the implementation, relatively low cost, easily soluble reactants ($Ca(NO_3)_2 \cdot 4\ H_2O$ and $(NH_4)_2HPO_4$) whose pH can be easily adjusted to keep the basicity of the reaction medium.

The syntheses were realized under nitrogen atmosphere, after previously vacuum was achieved in the installation, in order to avoid the presence of carbonate ion in the synthetic HA. The presence of this ion affects biocompatibility and also increases the solubility of the synthesized compounds (figure 1).

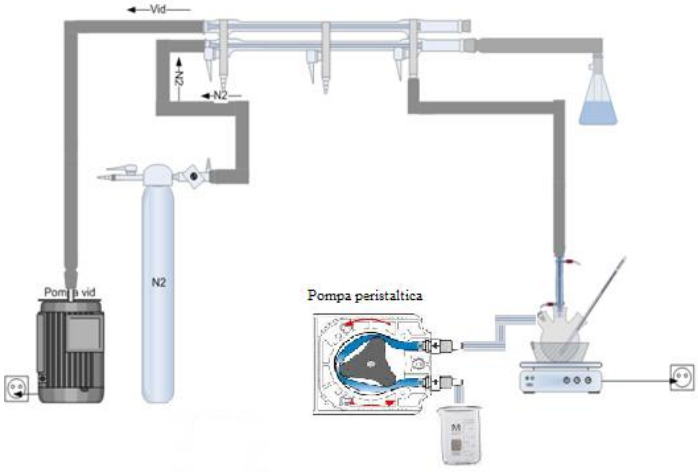


Figure 1. The experimental installation used for synthesis

Both hydroxyapatite and HA-AL compounds were characterized by FT-IR and X-ray diffraction[6].

As regards the analysis of XRD spectra obtained for both HA and HA-AL, the obtained spectra overlap with existing databases.

Since there are no shifts of the peaks in the diffraction pattern, it was concluded that HA powder obtained is pure and does not contain other phases (calcium phosphates), as seen in figure 2.

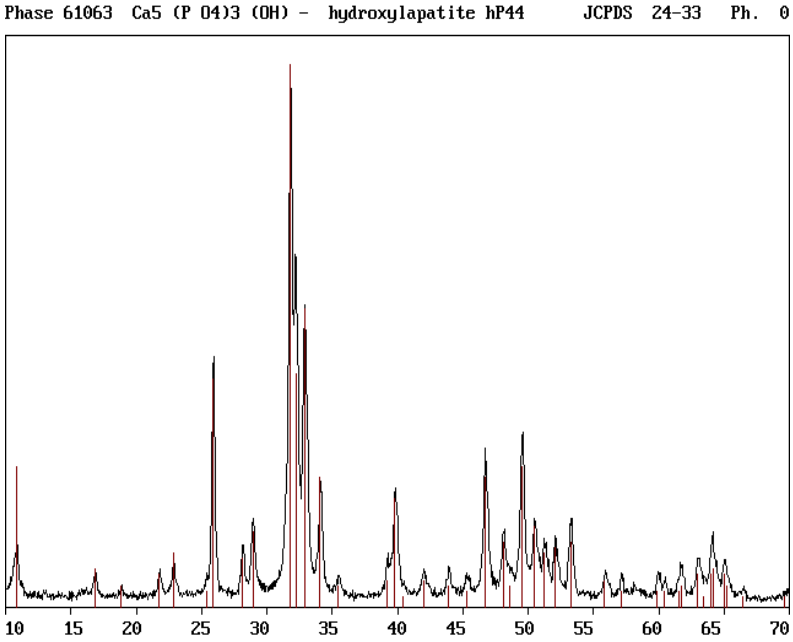


Figure 2. Diffraction spectrum of synthesized hydroxyapatite and position of the peaks corresponding to the commercial HA (red) from the database JCPDS

For all the synthesized compounds, regardless of the concentration of alendronate, in the FT-IR spectra were observed the presence of the characteristic peaks of alendronate.

The peak corresponding to N-H scissoring vibration at 1644 cm^{-1} is shifted (8-10 cm^{-1}) at 1636 cm^{-1} (HA-AL 10mM and 20mM), and at 1634 cm^{-1} (HA-AL 5 mM) as compared with the spectrum of HA-AL mechanical mixture 1:1, suggesting the occurrence of interactions between hydroxyapatite and alendronate (figure 3).

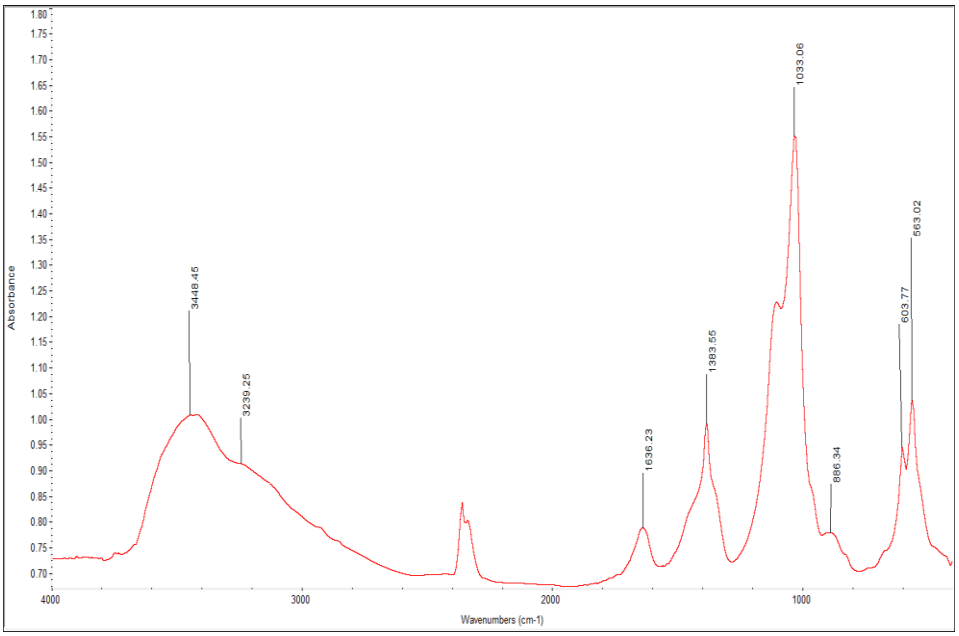


Figure 3. FT-IR spectrum of HA-AL 20 mM composite

It was also found the occurrence of a broad band in the range 3000 - 3600 cm^{-1} in the spectra of the compounds, the same band is present in the spectrum of alendronate.

HA-AL compounds shows at 1565 cm^{-1} the peak characteristic for stretching vibration of the -OH group (hydroxyapatite molecule) with an intensity more and more reduced to the complete disappearance of the spectrum of HA-AL 20 mM.

This may be due to the involvement of -OH groups in the formation of the bonds between hydroxyapatite and alendronate.

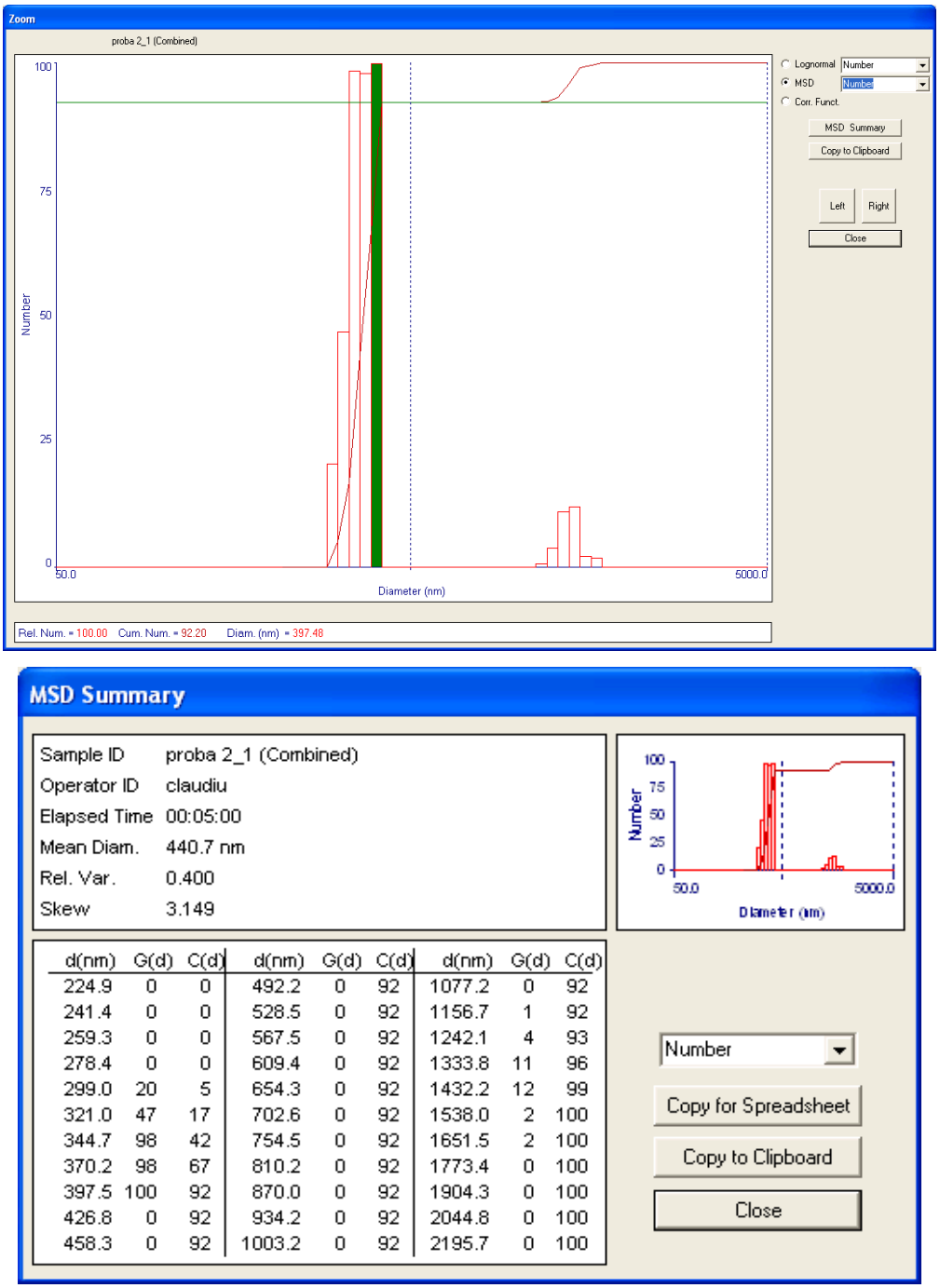
Thus, our studies are consistent with other studies in the literature, supporting the idea that between alendronate and hydroxyapatite there are certain interactions.

HA-AL dimensions were evaluated by dynamic light scattering measurements (DLS).

The results show that the HA-AL 20 mM synthesized particles have a particle size dimension in the range of [299-398] nm.

Graphs of particle size distribution on volume also indicates the presence of small agglomerates of larger particles with size in the range [1242-1651] nm.

Analyzing the two distributions (volume and number) was found that the maximum particle size is around 397 nm (figure 4).



d(nm)	G(d)	C(d)	d(nm)	G(d)	C(d)	d(nm)	G(d)	C(d)
224.9	0	0	492.2	0	92	1077.2	0	92
241.4	0	0	528.5	0	92	1156.7	1	92
259.3	0	0	567.5	0	92	1242.1	4	93
278.4	0	0	609.4	0	92	1333.8	11	96
299.0	20	5	654.3	0	92	1432.2	12	99
321.0	47	17	702.6	0	92	1538.0	2	100
344.7	98	42	754.5	0	92	1651.5	2	100
370.2	98	67	810.2	0	92	1773.4	0	100
397.5	100	92	870.0	0	92	1904.3	0	100
426.8	0	92	934.2	0	92	2044.8	0	100
458.3	0	92	1003.2	0	92	2195.7	0	100

Figure 4. Particle size distribution by number of hydroxyapatite-alendronate compound

A HPLC analytical method suitable for the determination of alendronate both in compounds and in liquid samples from the *in vitro* release studies was used[7].

FMOC was chosen as the derivatization reagent due to its superior properties compared to other reagents. It reacts under mild conditions with the amino group of alendronate, the resulting compounds being stable. The reaction mixture could be

injected into the non-polar column directly after the pH adjustment not being necessary to remove the excess of the reagent.

CHAPTER 5. HA-AL 20 mM COATING ON Ti SURFACES AND ITS CHARACTERIZATION

In chapter 5 we deposited hydroxylapatite-alendronate coatings on the metal part of the implant by pulsed laser evaporation (figure 5).

Figure 5. Titanium metal discs coated with a HA-AL 20 mM microfilm by MAPLE

For immobilization/deposition of the material on the metal surface of the collector Ti discs were used. Fixation of the material was done by MAPLE and the further characterization of the deposited films were carried out by AFM.

The thin films of material immobilized on the metal surface have a high adhesion on titanium substrate, pointing out the absence of delamination or any other morphological defects.

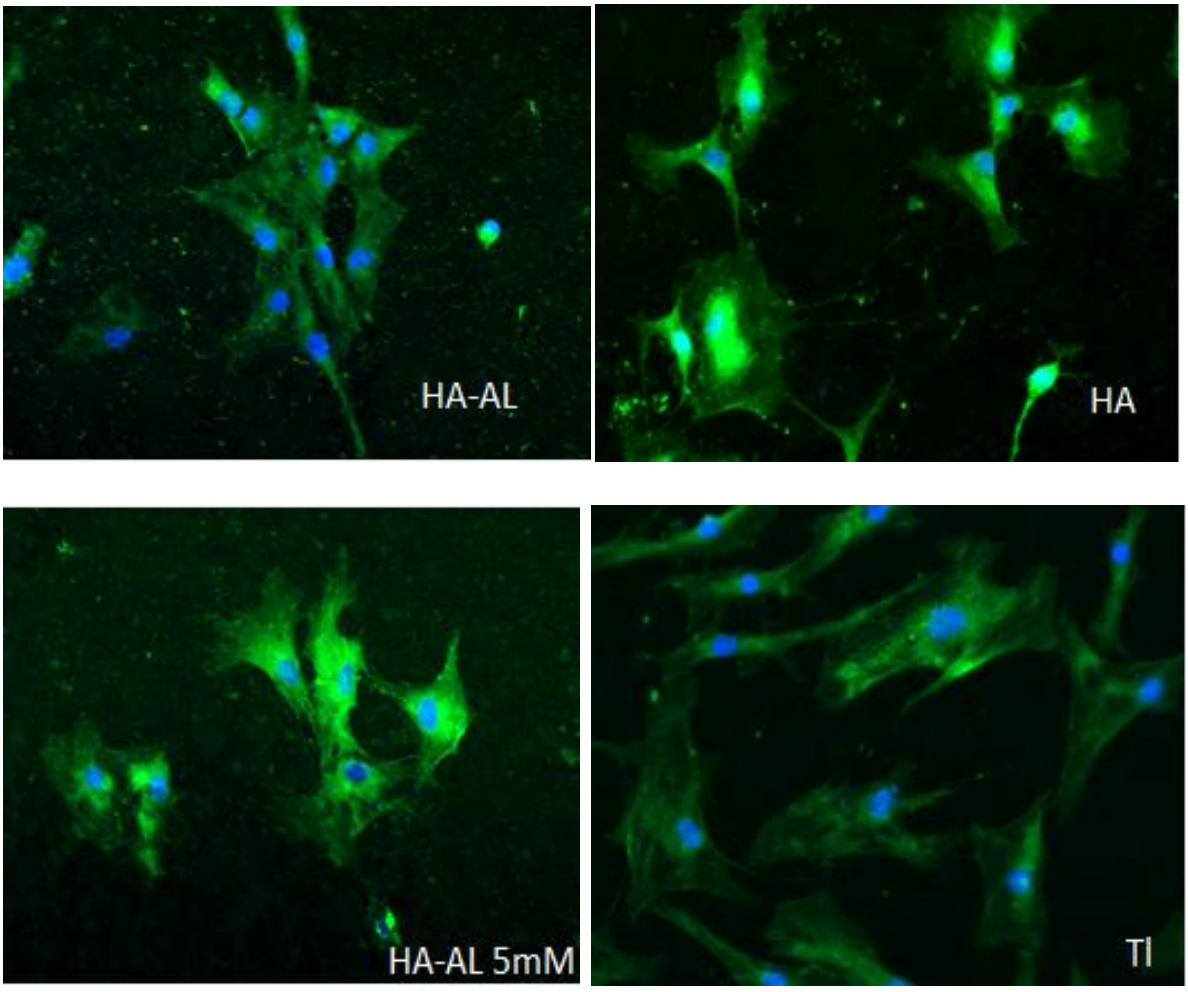
CHAPTER 6. BIOCOMPATIBILITY ANALYSIS OF SYNTHESIZED BIOMATERIALS

Biocompatibility of hydroxyapatite-alendronate synthesized composites was investigated in chapter 6.

A part of the study was dedicated to the investigation of the biocompatibility of materials based on hydroxyapatite-alendronate compounds.

MSC adhesion on the surface of biomaterials was evaluated by labeling of actin filament by immunofluorescence in order to analyze the organization of implantable cells on the surface at 72 hours after the attachment.

Cell biology experiments showed that both bone cells and human mesenchymal cells adhere and spread on all HA thin films deposited by MAPLE. The presence of alendronate in the films covering titanium increases the number of cell-biomaterial focal points (figure 6).



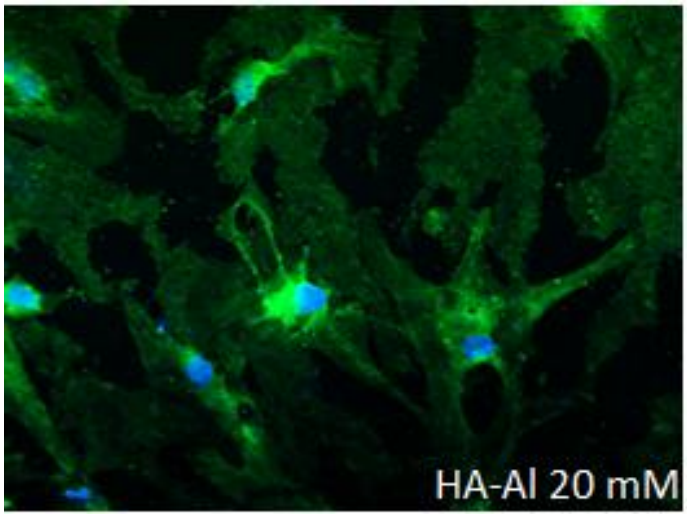


Figure 6. Immunofluorescence images of MSC cell adhesion to biomaterials: actin (green) was labeled with Alexa Fluor 488 conjugated phalloidin and nuclei are labeled with DAPI (blue) to identify cells (images 20 × overlapping channels).

Future studies are needed to demonstrate the functionality of HA-AL structures[8].

CHAPTER 7. IN VITRO RELEASE STUDIES OF ALENDRONATE FROM HA-AL COMPOSITES

In Chapter 7 we conducted a comparative study between the release of alendronate of the Ti disks coated with HA (and then subjected to a process of AL chemisorption from aqueous solutions of different concentrations) or with HA-AL compounds.

As a result of in vitro release studies it was found that crystalline hydroxyapatite is a stable phase in contact with the release medium and the alendronate linked to HA by synthesis is slow released of the metal component (titanium) in the release environment, over a period of 10 days. Thus, in the case of implantation may contribute to the resorption of bone, together with HA, that after implantation, produces chemical species that support adherence to the tissue surrounding the implant forming a conective structure.

Thus the synthesis of HA-AL composite can be considered viable to include bisphosphonate on the surface of metal components used in orthopedic prostheses.

SELECTIVE REFERENCES

[1] Puleo DA. Encyclopedia of Biomaterials and Biomedical Engineering (1), 2004, Marcel Dekker Inc., New York, 190-198.

[2] Wilson CJ, Clegg RE, Leavesley DI, Pearcy MJ. Mediation of biomaterial-cell interactions by adsorbed proteins: A review. Tissue Eng. 2005; 11: 1-18.

[3] Suratwala SJ, Cho SK, Van Raalte JJ, Park SH, Seo SW, Chang SS, Gardner TR, Lee FY. Enhancement of periprosthetic bone qwuality with topical hydroxyapatite-bisphosphonate composite. J. Bone Joint Surg. Am. 2008; 90:2189–2196.

[4] Papapoulos SE. Bisphosphonates: how do they work? Best Pract. Res. Cl. En. 2008; 22(5): 831–847.

[5] Knoch F, Eckhardt C, Alabre CI, Schneider E, Rubash HE, Shanbhag AS. Anabolic effects of bisphosphonates on peri-implant bone stock. Biomater. 2007; 28: 3549–3559.

[6] Gurgui-Ionescu C., **Balosache O.**, Mocanu A., Flori C., Moga S., Ducu C., Neamțu J. Studies concerning the shynthesis and characterisation of hydroxyapatite powder. Acta Medica Marisiensis. 57(2):90-93, 2011

[7] **Balosache O.E.,** Bubulică M.V., Popescu M., Manda C.V., Croitoru O.,Băniceru M., Melinte R., Neamțu J. Synthesis physico-chemical characterization of a hydroxiapatite-alendronte composite. Current Health Sciences Journal. 39(supl. 3): 39-43, 2013.

[8] Colnot C., Skeletal cell fate decisions within periosteum and bone marrow during bone regeneration, Journal of Bone and Mineral Research, 24 (2):274-282, 2009.