RIGA TECHICAL UNIVERSITY

Faculty of Materials Science and Applied Chemistry
Institute of General Chemical Engineering

Arita DUBŅIKA
Doctoral student in program „ Chemical Engineering”

CONTROLLED RELEASE DRUG DELIVERY SYSTEMS BASED ON SILVER DOPED HYDROXYAPATITE

Summary of Doctoral Thesis

Scientific Supervisors
Professor, Dr. sc. ing.
L.BĒRZIŅA-CIMDIŅA
Docent, Dr. sc. ing.
D.LOČA

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OFFICIAL REVIEWERS

Associate professor, Dr.habil.sc.ing. Visvaldis Švinka
Institute of Silicate Materials, Riga Technical University

Professor, Dr.-Ing. habil. Aldo R. Boccaccini
Institute of Biomaterials, Friedrich-Alexander-Universität Erlangen-Nürnberg, Germany

Professor, D.D.S., M.Sc., Dip.prosth. (Toronto), Dr.habil.med., Pēteris Apse
Faculty of Dentistry, Riga Stradiņš University

CONFIRMATION

I confirm that I have developed the present Doctoral Thesis, which is submitted for consideration at Riga Technical University for scientific degree of the doctor of engineering sciences. The Doctoral Thesis has not been submitted at any other university for the acquisition of a scientific degree.

Arita Dubņika …….

Date: ……

The Doctoral Thesis is written in Latvian language, it contains Introduction, 3 chapters - Review of Literature, Methods, Discussion of Experiments, Conclusions, list of References, as well as 74 illustrations, 21 tables and 2 annexes. 175 references are used for this Doctoral thesis.

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„Love has greater value than any nuclear science or astronomy, and love is the hardest to leave, prove or code. Love has no sign of its own. Love is too large for all signs and every sign."
(Epiphanies, Imants Ziedonis)

CONTENTS

OVERWIEV OF THE DOCTORAL THESIS

State of the art and novelty

Certain structure and size of bones serve as a precondition for ensuring and preserving the quality of human life. Bones are the foundation of two important survival functions: firstly, the ability to ingest food and, secondly, to move. The healthiness of skeletal bones in human life determines the ability to move, but the condition of jawbones and teeth is responsible for food intake, verbal functions and ensuring of psycho emotional comfort. Many bone pathologies and injuries are treated with surgical methods and different implant materials, therefore development of multifunctional biomaterials are of great significance in improving and renewing functions of hard tissues.

Across the world, different calcium phosphate biomaterials are used, including ceramics and cements, as well as bio-glass, glass ceramics and coatings for metallic implant materials. Calcium phosphate biomaterials possess high bioactivity and osteoconductivity, therefore these materials create strong bonds with healthy bone tissue. Some of calcium phosphate biomaterials are bioresorbable – with time they are absorbed by the body, do not create toxic substances, and allow healthy tissues to replace implant material. The only disadvantage of calcium phosphate biomaterials is low mechanical endurance; therefore they are used in non-load bearing applications, mainly in maxillofacial surgery, as well as for treating osteoporosis.

In medicine one of the most widespread calcium phosphate biomaterials is hydroxyapatite, which is a mineral phase of bone. By adding antibacterial effect to hydroxyapatite properties and creating controlled drug delivery systems based on this material, it is possible to obtain multifunctional material. The obtained material would be not only bioactive and osteoconductive, but also antibacterial, and would release controlled amount of drugs in post-surgery period, thereby preventing the risk of possible infections.

The structure of hydroxyapatite can be easily modified by replacing calcium, phosphate or hydroxyl ions with other ions. The antibacterial effect of silver ions has been known for centuries; therefore introduction of silver ions into hydroxyapatite structure would grant antibacterial properties to implant material. Moreover, hydroxyapatite is suitable for controlled delivery of biologically active substances in site specific location. It is possible to form hydroxyapatite as mesoporous material, in which it is possible to introduce biologically active substances by using chemical modifications or impregnation technologies. For such materials it is possible to additionally create polymer coatings that would modify the active

substance release kinetics. Improving the structure of the material and methodology of introducing biologically active substances, it is possible to control the active substance *in vitro* release.

The aim of the Doctoral Thesis

To develop technological scheme for obtaining silver-doped hydroxyapatite local drug delivery systems and to study the properties of the obtained products.

Tasks set to realize the aim of the Doctoral Thesis

- to determine significant parameters for the preparation of silver-doped hydroxyapatite and controlled drug delivery systems on its basis. Following the obtained results, to develop technological scheme for the development of silver doped hydroxyapatite;
- to develop necessary analytical methods for the evaluation of obtained product properties;
- to determine the release kinetics of active substances and silver ions in simulated body fluids;
- to evaluate the biocompatibility of prepared composites and bioavailability of active substances.

Scientific significance of the Doctoral Thesis

- for the first time properties of silver doped hydroxyapatite depending on the preparation method have been researched and porous silver doped hydroxyapatite scaffolds have been obtained;
- for the first time controlled release drug delivery systems based on the antibacterial materials have been obtained;
- the influence of silver doped hydroxyapatite composition and its modifications on the release kinetics of different biologically active substances has been evaluated.

Practical significance of the Doctoral Thesis

- technological scheme for obtaining silver doped hydroxyapatite with controllable physical and antibacterial properties has been developed;
- methods for the preparation of controlled release drug delivery systems based on bioactive implant materials with antibacterial properties have been developed.

Approbation of the Doctoral Thesis

The scientific achievements and main results of the scientific research of this Thesis have been presented in 12 international conferences, summarized in 7 full text scientific manuscripts and 18 peer-reviewed conference proceeding abstracts, and also one collective monograph has been submitted for the publication in book “Hydroxyapatite (HAP) for biomedical applications”.

REWIEW OF THE LITERATURE

Every year the number of surgical procedures where calcium phosphate implant materials are used is increasing. Researches in hydroxyapatite implant materials possess great importance for further developments in maxillofacial and oral surgery in order to ease the pain and renew the functionality of damaged bone tissue, as well as to ensure positive outcome of such surgical procedures. Similarities between hydroxyapatite and mineralogical structure of the bone ensure the biocompatibility of implant material. In surgery where human tissue comes into contact with implant materials, there is an increased risk of inflammation, therefore, to ensure the needed biological interaction, it is necessary to develop functionalized implant materials.

It is possible to include different ions into hydroxyapatite structure to improve its properties. By including silver ions into hydroxyapatite structure, antibacterial activity of the material can be ensured. To obtain silver-doped hydroxyapatite, different methods have been proposed. Literature review shows variable phase content for thermally treated and thermally non-treated samples, therefore deeper investigations into the properties of this material is needed to determine the efficacy of antibacterial activity. Additionally, material properties need to be evaluated based on the material preparation method, since different literature sources point out different properties of silver-doped hydroxyapatite. Due to the fact that hydroxyapatite does not dissolve in human body, it is also important to evaluate its cytotoxicity, especially in silver-doped samples. Literature summarizes researches of *in vitro* silver dissolution from silver doped hydroxyapatite powders for the period of only 3 to 14 days. Implant material will stay in the human body for a prolonged period of time, therefore it is necessary to evaluate silver ion dissolution from the samples for a period of at least two to three months. Properties of the obtained implant material have significant implications for its further use in the development of controlled drug delivery systems.

Different drugs are used to improve the bone tissue regeneration, but most often in maxillofacial and oral surgery dexamethasone sodium phosphate and lidocaine hydrochloride are used to reduce swelling and for analgesic purposes. Each drug delivery method has its advantages and disadvantages, therefore drug delivery methods are constantly developing and being modified or improved. The most important aspect of local drug delivery is to ensure the concentration of the specific active substance for a specific period of time. Wide researches into development of controlled drug delivery systems based on hydroxyapatite were started only within the last decade, therefore it is still an ongoing process to look for the optimal drug delivery conditions to control the drug release. To ensure controlled drug release, it has been noted that the most effective strategies are to provide the polymer coatings and chemical binding of drugs on the different interfaces. Many methods are based on polymer crosslinking, using crosslinking agents that can be toxic, therefore their application for implant

materials is limited. Due to this fact it is necessary to develop the natural polymer coatings without crosslinking agents, and to evaluate the effect of crosslinking agent on the drug release kinetics. Chemical drug binding methods reviewed in the literature mostly describes the drug binding to the polymer scaffolds. These methods need to be transferred to silver doped hydroxyapatite scaffolds, by creating polymer coatings to which molecules of pharmaceutically active substances can bound. Schematic summary of the drug delivery systems preparation strategies is shown in Figure 1.

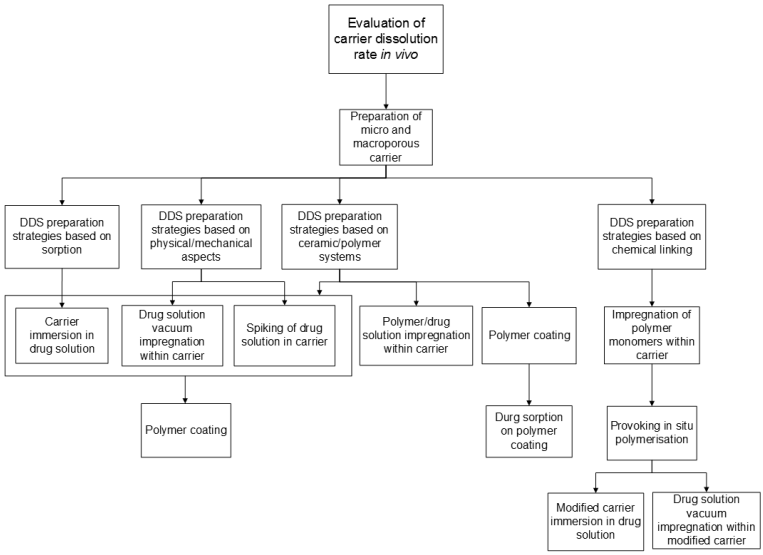


Fig.1. Schematic summary of drug delivery systems preparation strategies

Not only drug release rate for obtained systems should be evaluated, but according to the literature, its connection with porosity of the scaffold, processing technology and morphology should be evaluated. Analytical methods to determine drug content is of great significance in order to diagnose small concentration of drugs and whether or not the drug has been fully released.

As a result of the literature review, it has been concluded that up till now controlled drug delivery systems based on silver doped hydroxyapatite have not been researched. Therefore the research done in the framework of this Doctoral Thesis opens wide possibilities for obtaining materials that can simultaneously ensure the therapeutic effect of drugs during 3 to 5 days after the surgery and a prolonged antibacterial effect due to silver ions.

EXPERIMENTAL METHODS

The experimental process scheme of Doctoral thesis and determined parameters are shown in Figure 2.

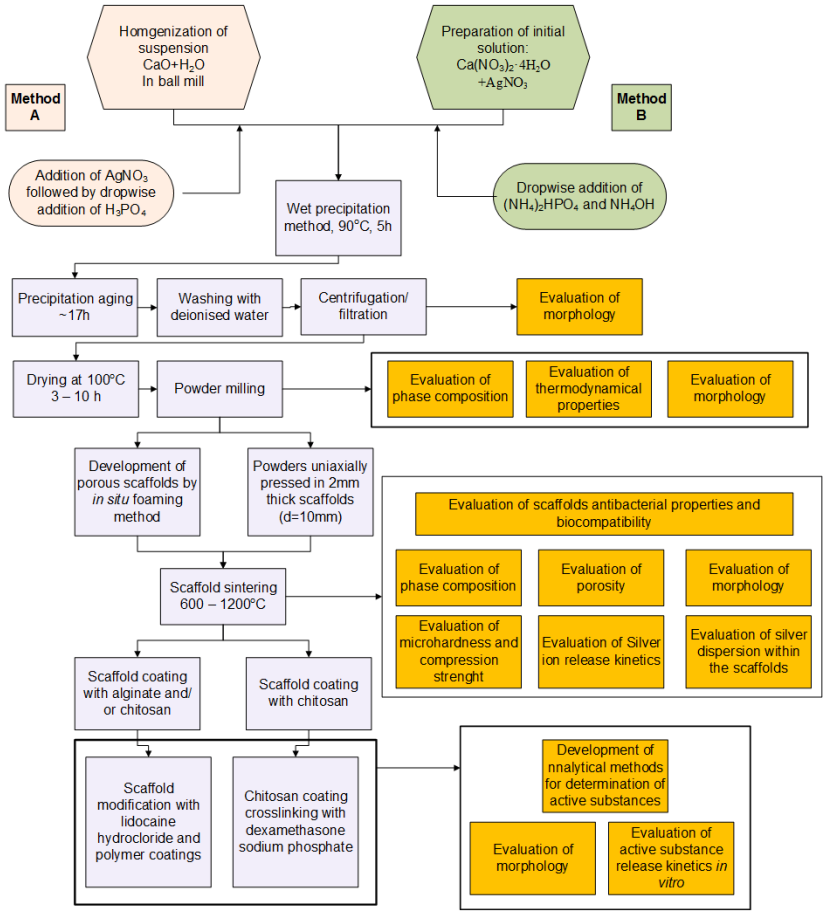


Fig.2. Sample preparation process and investigated properties

Silver doped hydroxyapatite development and evaluation

Two types of chemical precipitation methods with different reagents were used to obtain silver doped hydroxyapatite (HAp/Ag). Reaction was carried out at 90 ^{o}C and obtained precipitate was aged for 17 h. To remove the unreacted ions synthesis products were washed with deionised water. During reaction and after it suspension samples were dried at 100 ^{o}C and the morphological changes of particles were analyzed using a field emission

scanning electron microscope (SEM, Tescan, Mira/LMU). Obtained suspensions were centrifuged or filtered and dried at 100 ^{o}C for 3 – 10 h. Phase composition of obtained powders was evaluated using the Fourier transform infrared spectroscopy analysis (FT-IR, Varian 800 FT-IR Scimitar Series), X-ray powder diffraction (XRD, PANalytical X-Pert Pro) and X-ray fluorescence spectrometry (XRF, Bruker Pioneer S4) analysis. Thermal properties were measured by optical dilatometry (EMO-1750/30-K) and differential thermal analysis (DTA, BÄHR „DTA703"). The sample specific surface area was evaluated using nitrogen sorbtometry with Brenauer-Emmett-Teller method (BET, Quantachrome QuadraSorb SI).

Development and evaluation of silver doped hydroxyapatite scaffolds

In situ foaming method was used to form porous scaffolds from obtained powder materials (pore forming agent NH_4HCO_3), for obtaining dense scaffolds powders were uniaxially pressed with force 2 – 15 kN. Samples were thermally treated at 600 to 1000 ^{o}C temperature 1 - 2 hours.

Phase composition with XRD, XRF and FTIR was evaluated for all obtained samples, Archimedes method was used for the determination of sample porosity, silver ions release kinetics was determined with electrothermal atomic absorption spectrometry (ETAAS, AANALYST 600, PerkinElmer), scaffolds morphology was evaluated with SEM and sample specific surface area was evaluated with BET. The compressive strength of the porous scaffolds was determined using a Zwick/Roell universal testing machine. For the dense scaffolds Vickers microhardness was evaluated (Vickers M41), antibacterial properties were determined against two type of bacteria *Ps.aeruginosa* ATCC 27853 and *S.epidermidis* ATCC 12228.

Modification of scaffolds with chitosan and dexamethasone sodium phosphate

Dense and porous scaffolds impregnated in vacuum and isostatically pressed in 4 wt% chitosan 5% acetic acid water solution. Dried scaffolds were washed with 1M NaOH water solution, dichloromethane and dimethylformamide. Scaffolds repeatedly dried and vacuum impregnated with dexamethasone sodium phosphate in dimethylformamide solution and isostatically pressed in dexamethasone solution by adding N,N-diisopropylethylamine as catalyst

Scaffolds morphology was evaluated using SEM, drug release kinetics in simulated body fluid (SBF) was determined with ultra performance liquid chromatography method (UPLC, Waters Acquity UPLC).

Modification of scaffolds with alginate/chitosan and lidocaine hydrochloride

HAp and HAp/Ag porous ceramic scaffolds were impregnated with lidocaine hydrochloride water solution (60 mg/mL) and polymer/water solution by using vacuum impregnation method. HAp/Ag alginate/lidocaine/alginate scaffolds for alginate crosslinking were placed in

30% $CaCl_2$ water solution for 4 h. The type of polymers and order of coatings on the scaffolds are shown in figure 3.

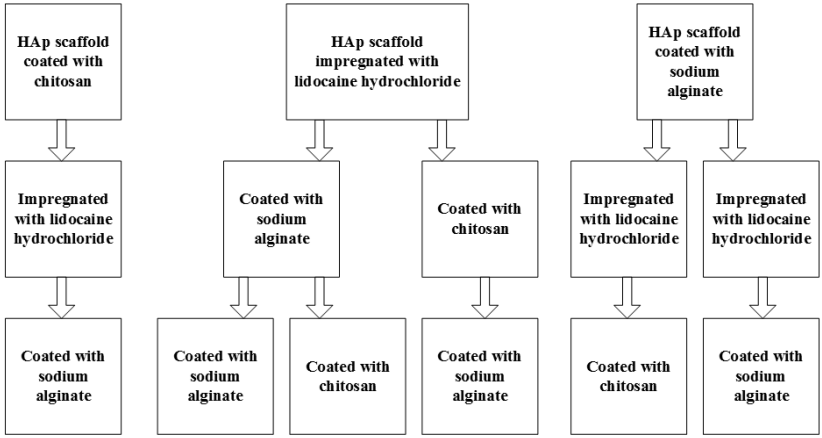


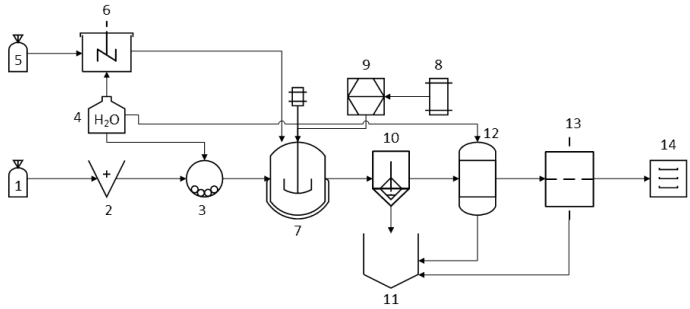
Fig.3. Preparation of lidocaine delivery systems on porous ceramic scaffolds

Scaffolds morphology was analyzed with SEM and drug release kinetics was determined in phosphate buffer solution (PBS) using high performance liquid chromatography (HPLC, Waters 2695 Alliance Separations Module).

DISCUSSION OF EXPERIMENTS

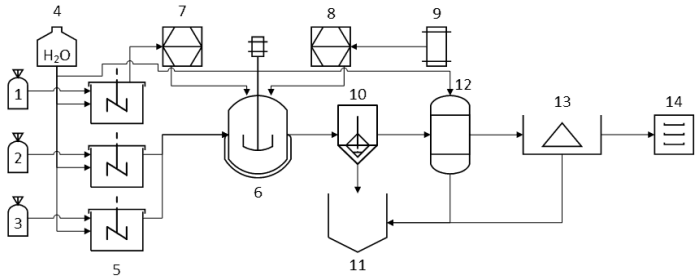
1. Development of HAp/Ag preparation methods and technological schemes

Wet chemical precipitation methods based on the precipitation of ions within the water solutions were selected for the preparation of HAp/Ag. Prescribed methods were transferred from reactors with 0,2 L volume to reactors with 2,0 L volume to obtain the greater outcome and therefore more possibilities to test and use the material. All technological parameters of methods described in literature were modified due to the necessity of up scaling the synthesis. Technological schemes of HAp/Ag preparation proceses were developed. In the case of method A – CaO, H_3PO_4, $AgNO_3$ and in the case of method B - $Ca(NO_3)_2$,$(NH_4)_2HPO_4$, HN_4OH, $AgNO_3$ were used as precursors (see fig.4. and fig.5.).



1 – CaO, 2 – conical crusher, 3 – planetary ball mill, 4 – deionized water tank, 5 – $AgNO_3$, 6 – dissolution vessel with mixer, 7 – thermostatically controlled reactor with mechanical mixer and pH sensor, 8 – 2M H_3PO_4, 9 – dosing device, 10 – settling vessel, 11 – separated solutions container, 12 – rinsing tank with pH sensor, 13 – filtration equipment, 14 – drying oven.

Fig.4. Technological scheme of HAp/Ag A preparation method



1 – $(NH_4)_2HPO_4$, 2 – $AgNO_3$, 3 – $Ca(NO_3)_2$, 4 – deionized water tank, 5 – dissolution vessels with mixers, 6 – thermostatically controlled reactor with mechanical mixer and pH sensor, 7, 8 – dosing devices, 9 – NH_4OH solution, 10 – settling vessel, 11 – separated solutions container, 12 – rinsing tank with pH sensor, 13 – centrifuge, 14 – drying oven.

Fig.5. Technological scheme of HAp/Ag B preparation method

2. Analysis of thermally untreated HAp/Ag samples

Eight A method synthesis and four B method synthesis with different silver content in samples were carried out by using the developed technological schemes. Each synthesis was repeated at least 3 times. For obtained materials such properties were determined:

1) phase composition – to approve the Ag^+ ion incorporation in the structure instead of byproduct formation;

2) incorporated silver content in the structure – to determine the efficiency of silver ion incorporation in the HAp structure;

3) BET specific surface area and particle morphology – to determine size and shape of the particles and anticipate properties of thermally treated samples;

4) thermodynamical properties – to determine the thermal stability and optimal thermal treatment conditions.

Obtained powder sample XRD patterns are represented at fig.6.

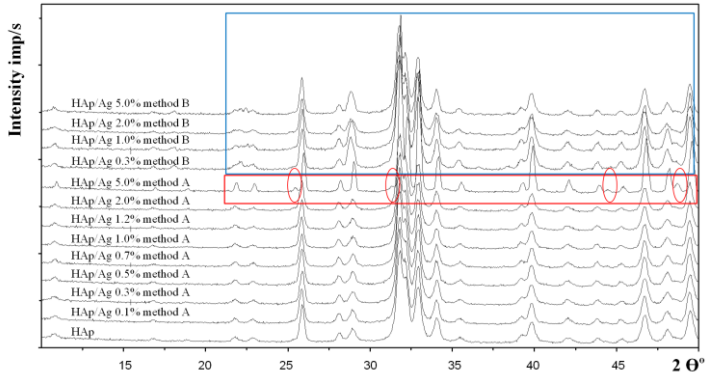


Fig.6. XRD patterns of A and B preparation method HAp/Ag and HAp thermally untreated powder samples

Obtained powder sample XRD patterns correspond to hexagonal crystal structure of HAp phase, suggesting that silver ions are incorporated in the HAp structure. Reaching the silver content 5.0% β-tricalcium phosphate (β-$Ca_3(PO_4)_2$) phase was detected for the samples prepared by method A.

Calculated unit cell a and c parameters approved silver ion incorporation in the structure as they were increasing with incorporation of silver ions. These results correspond to the literature, approving silver ion incorporation.

Incorporated silver amount (wt%) in HAp structure was detected for powder samples. Silver ion incorporation efficiency in HAp structure decreased by increasing the theoretically added silver amount. In the case of method A efficiency of silver ion incorporation decreased from 99 ± 1% for

samples with 0,1% theoretical silver amount to 50 ± 2% for samples with 5.0% theoretical silver amount. In the case of method B faster decrease of silver recovery was detected. For the samples with 0.3% theoretical silver amount the recovery reached 50 ± 2% but for samples with 2,0% theoretical silver amount the recovery decreased to 15 ± 4%. Incorporation efficiency of silver ions indicates that the other byproducts are formed.

It was observed that BET specific surface area decreased by increasing the added silver amount (see fig.7.). The decrease of BET specific surface area can be explained by the changes of HAp/Ag unit cells.

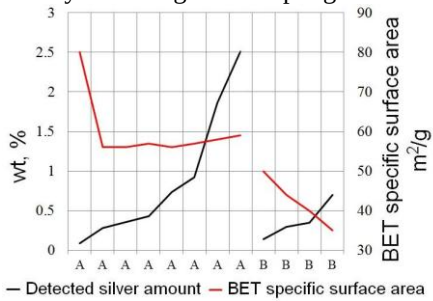


Fig.7. HAp/Ag specific surface area and incorporated silver amount

Optical dilatometry results showed the structural changes of HAp/Ag. It was determined that sintering temperature for HAp/Ag samples, prepared by method A, is 150 ± 10 ^{o}C higher than that for pure HAp. Sintering temperature for HAp/Ag samples prepared by method B was increased by the increase of incorporated silver amount within the structure. Incorporation of 0.14 wt% of silver ions resulted in the sintering temperature increase by 50 ± 5 ^{o}C but incorporation of 0.70 wt% of silver ions, increased the sintering temperature difference between HAp/Ag and HAp by more than 200 ± 10 ^{o}C. These results correspond to BET specific surface area results, indicating that HAp/Ag powder particle size increases by increasing incorporated silver amount in the case of synthesis method B, therefore compression of particles occurs slower and sintering temperature increases during the thermal treatment.

3. Analysis of thermally treated HAp/Ag powder samples

Morphological changes of HAp/Ag powder samples during the sintering process are showed in figure 8. HAp/Ag particles have needle like morphology and the length of the particles is in the range from 20 to 65 nm. The length of the particles sintered at 1000 ^{o}C for 2 h increased and was in the range of 70 to 200 nm, indicating the grain growth. Densification of particles can be observed for samples sintered at 1150 ^{o}C for 2 h.

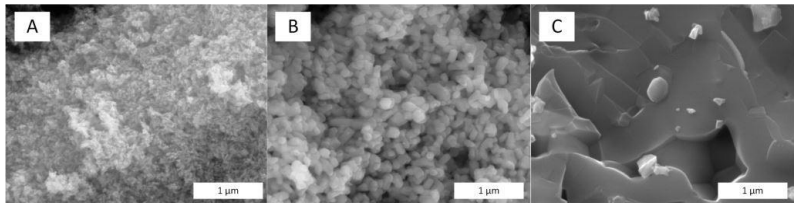


Fig.8. SEM images of A method HAp/Ag particles: A– unsintered powder; B– sintered powder at1000 ^{o}C 2h; C– sintered powder at 1150 ^{o}C 2h

Analysis of the scaffold morphology and phase composition after the thermal treatment was carried out. It was determined that silver evaporates form the dense scaffold surface during the sintering process. The phase composition changes can be observed not only by the change of thermal treatment temperature, but also depending on the HAp/Ag preparation method. Comparison of the phase composition and its changes in different thermal treatment temperatures for powder samples synthesized by both preparation methods is summarized in table 1.

Table 1

Phase composition of HAp/Ag samples after the thermal treatment

HAp/Ag preparation method	500 ^{o}C	700 ^{o}C	900 ^{o}C	1000 ^{o}C	1150 ^{o}C
A	HAp	HAp	HAp AgO	HAp AgO Ag	HAp AgO Ag
B	HAp	HAp Ag	HAp Ag AgO	HAp AgO	HAp AgO

4. Microhardness evaluation of dense scaffolds

Comparing the dense scaffold microhardness after the thermal treatment at 1000 and 1150 ^{o}C temperature it was observed that the applied force during the sample preparation affects the sample microhardness (see fig.9).

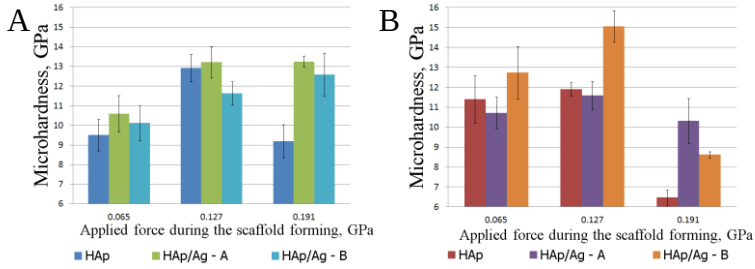


Fig.9. HAp and HAp/Ag dense scaffold microhardness after the thermal treatment at 1000 ^{o}C (A) and 1150 ^{o}C (B) depending on the applied force during the sample formation

If the applied force at the formation process of dense scaffolds was increased from 0.065 to 0.127 GPa, sample microhardness increased. Increasing the applied force up to 0.191 GPa, decrease of sample microhardness was observed. Using the higher applied force in the sample formation process particles are packed. If the particle packing efficiency level is exceeded, in this case at higher applied forces, deformation of particles occurs. Due to that the contact surface between particles is deformed and the microhardness decreases. It was determined that within the error limits the optimal applied force during the formation process is 0.127 GPa, ensuring the highest microhardness for both types of HAp/Ag dense scaffolds that are thermally treated at 1000 or 1150 ^{o}C temperature.

5. Effect of thermal treatment temperature on the properties of HAp/Ag porous scaffolds

SEM images of HAp/Ag porous scaffolds showed that scaffolds prepared from HAp/Ag A method powder contain more micro and nano pores comparing to the scaffolds that are prepared from HAp/Ag B method powder. The pore surfaces are smoother and the pore walls are denser for B method HAp/Ag porous scaffolds (see fig.10).

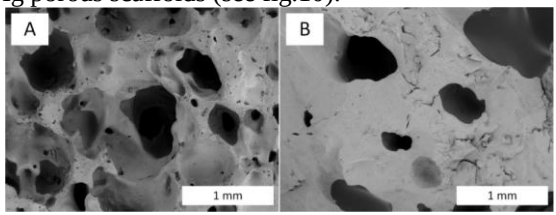


Fig.10. Thermally treated porous samples: A – prepared from A method powder, B – prepared form B method powder

For the both types of HAp/Ag ceramic scaffolds, silver containing particles can be observed within the pore walls. In the case of powder preparation method A silver containing particles were observed on the pore surfaces. During the thermal treatment, silver is subjected to the reversible reaction $2Ag + O_2 \leftrightarrows 2AgO$. Silver containing particles that are on the pore surface are with polyhedron-like crystal structure that was deformed by increasing the thermal treatment temperature (see fig.11).

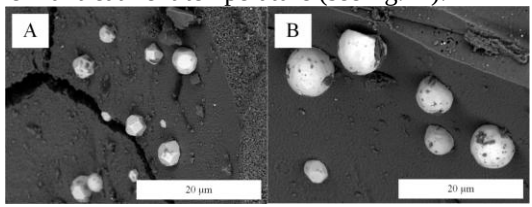


Fig.11. Sintered HAp/Ag A method porous scaffolds: A – 1000 ^{o}C, B – 1150 ^{o}C

For porous HAp/Ag scaffolds the expected correlation between density and compressive strength was noticed. Denser structure, respectively, for the samples treated at 1200 ^{o}C has higher mechanical strength. Increasing the sintering temperature from 1000 ^{o}C up to 1200 ^{o}C it is possible to obtain samples with 2–4 times higher compressive strength. Porous HAp/Ag scaffolds with 0.5 – 0.8 wt% silver content have 1.3 – 2.1 times lower mechanical strength than HAp porous scaffolds (see fig.12.). One of the reasons for the compressive strength reduction could be Ca^{2+} ion substation with Ag^{+} ion, because silver ion Ag^{+} (1.28 Å) is larger than calcium ion Ca^{2+} (0.99 Å) leading to the possible deformation of HAp crystal structure. XRD and SEM investigations indicated that Ag containing particles were formed on the sample surface during the thermal treatment, leaving vacancies in the HAp structure responsible for the lower mechanical properties.

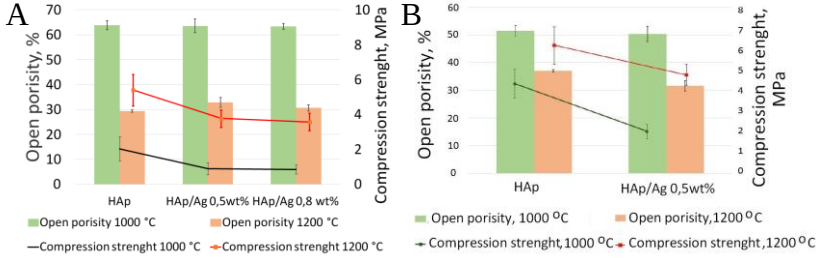


Fig.12. Compressive strength of HAp/Ag porous scaffolds: A – powder preparation method A, B – powder preparation method B.

6. Silver ion release kinetics *in vitro*

Antibacterial properties of silver containing materials depend on the silver amount in the material as well as from the phase composition and the dissolution rate of the material. Silver ion release was determined in the simulated body fluid (SBF) for one year to evaluate the silver ion release from the obtained materials.

Comparison of the silver release in the SBF from dense and porous scaffolds that were heat treated at 1000 ^{o}C temperature for 2 h is showed in figure 13.

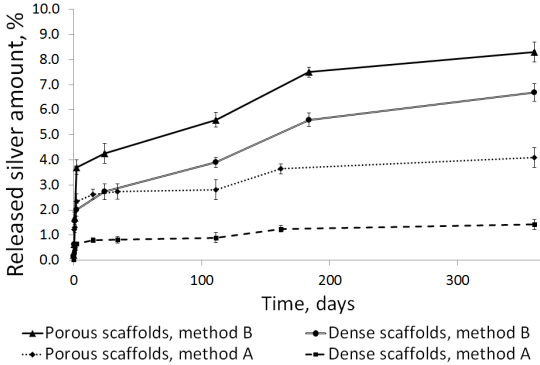


Fig.13. *In vitro* silver ion release kinetics from HAp/Ag scaffolds

Obtained curves of silver ion release kinetics showed that the silver ion release rate in the first 2 incubation days is two times higher for scaffolds that were prepared form calcium nitrate, ammonium hydrogenphosphate, silver nitrate and ammonium (method B) comparing with scaffolds prepared from HAp/Ag A method powder. After the first 2 days silver ion release rate stabilized and for the next three months from both types of dense and porous HAp/Ag scaffolds, Ag^+ ions were released with a rate of 0.001 ± 0.0005wt%/h from the incorporated silver amount in the scaffolds. Using the HAp/Ag scaffolds with 1 wt% Ag amount, silver ion concentration would be below 0.025 ± 0.008 μg/L per hour. Based on the literature data such silver ion concentration is not toxic to the human body.

SEM images of HAp/Ag scaffold morphology after 12 month incubation period in SBF are shown in figure 14. HAp layer was observed on the scaffold surface and porous scaffold cross-section. If the HAp layer is formed on the material surface in the SBF solution, the material would be bioactive in the *in vivo* experiments.

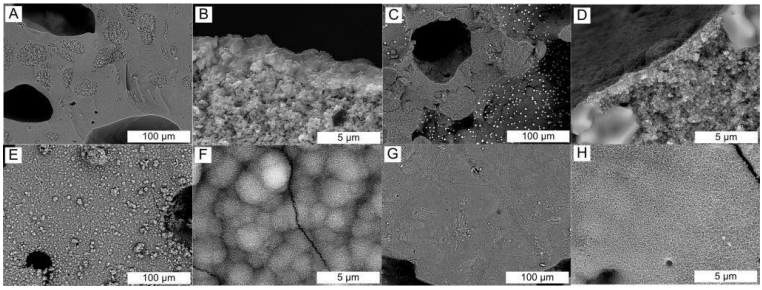


Fig.14. Microstructure of HAp/Ag scaffolds after an incubation in SBF for one year: A,B – cross-section of B method scaffold, C,D – cross-section of A method scaffold, E,F – surface of B method scaffold, G,H – surface of A method scaffold

7. Evaluation of HAp/Ag scaffold antibacterial properties

To determine the optimal preparation and processing parameters for the development of antibacterial porous scaffolds, antibacterial properties depending on the sample thermal treatment temperature, silver amount and HAp/Ag preparation method were evaluated. In the study antibacterial properties were evaluated against *gram* positive *S. epidermidis* and *gram* negative *P. aeruginosa* bacterial strains.

The samples containing pure HAp did not demonstrate any antibacterial activity. The colonization activity of both bacteria strains was inhibited on the Ag-containing samples, especially on the samples prepared by method A with a silver amount of 1.86 wt% (see fig.15.). Only rare and separate *S. epidermidis* cells with the absence of glycocalyx were found on the surface of Ag containing biomaterials.

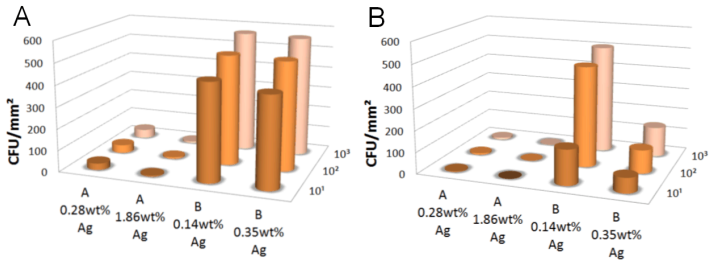


Fig.15. Colonization intensity of: A – *S. epidermidis*, B – *Ps. aeruginosa*

The colonization results of bacterial strains approved that the antibacterial activity of HAp/Ag depends on the silver content and the thermal treatment temperature (see fig.16.). The colonization activity of *S. epidermidis* was inhibited on the silver-containing samples, especially on the samples sintered at 1150 °C. By increasing the scaffold thermal treatment temperature scaffold porosity decreases. As a result it is harder for bacteria to adhere and spread on the scaffold surface.

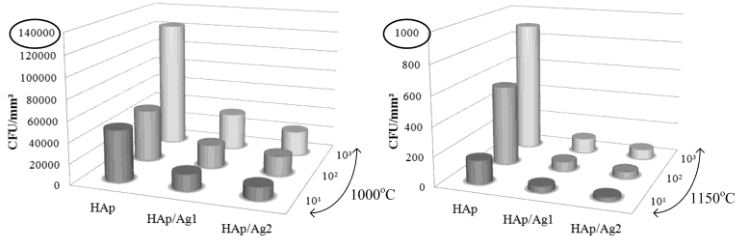


FIg.16. Colonization intensity of *S.epidermidis* on the scaffolds prepared from HAp/Ag A method powder: HAp/Ag1 - Ag silver content 0.2%; HAp/Ag2 - Ag silver content 1.1%.

From all antibacterial activity results it can be concluded that samples prepared by method A containing 1.1 wt% silver and treated at 1150 ^{o}C temperature show the best antibacterial properties towards *gram* positive (*S. epidermidis)* and *gram* negative (*Ps. aeruginosa)* bacterial strains.

8. Lidocaine hydrochloride delivery systems on the basis of hydroxyapatite

Early studies for drug delivery system development were made with HAp porous scaffolds. Scaffolds were coated with alginate and/or chitosan coatings and impregnated with lidocaine in various orders (see fig.3.).

FTIR analysis of obtained composite materials indicated that no chemical reaction between HAp, lidocaine and alginate and/or chitosan occurs during the impregnation process. However SEM micrographs show that the crystalline structure of lidocaine varies, depending on the order of polymer and lidocaine impregnation (see fig.17).

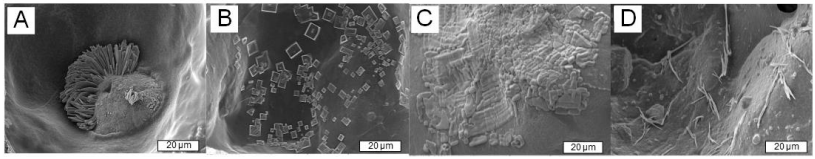


Fig.17. SEM microphotographs: A – HAp/chitosan/lidocaine; B – HAp/alginate/lidocaine; C – HAp/alginate/lidocaine/alginate; D – HAp/alginate/lidocaine/chitosan

Crystalline structure of lidocaine was affected by the development of crystallization centers and the lidocaine solubility in the polymer solutions during the impregnation process. During the modification process lidocaine was repeatedly dissolved and recrystallized, therefore the structure of lidocaine changed and in the presence of vacuum, it could be deformed. The water evaporation process occurred slower, if the more viscous chitosan solution was used, and lidocaine crystal growth could occur in the one place with larger concentration.

Crystal morphology of lidocaine and its placement in the scaffold affected its release rate. In order to evaluate the lidocaine release kinetics, composites were placed in PBS for 148 h. Released lidocaine amount was detected during the incubation (37 °C, 50 rpm) period (see fig.18).

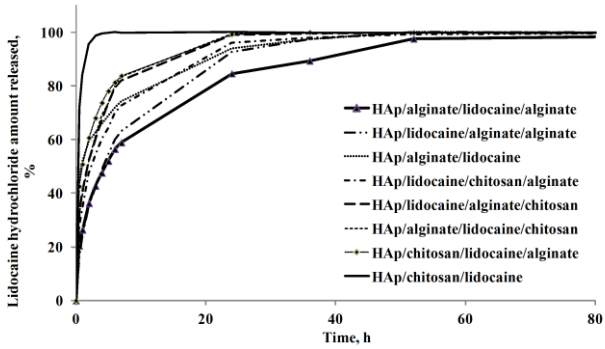


Fig.18. Lidocaine hydrochloride release kinetics from HAp/polymer/drug composites

For composites coated with chitosan in the first hour the initial burst release up to 70% was observed. Scaffolds coated with alginate showed the initial burst release between 16% and 30%. During the incubation period the slowest lidocaine release was observed for HAp/alginate/lidocaine/alginate composites where lidocaine release was sustained even up to 60 h, while from the HAp/chitosan/lidocaine scaffold complete lidocaine release was observed already in the first 6 h.

Controlled release of lidocaine for 60 h is long enough to ensure the analgesic effect in the first days after the surgery. Due to that the study with HAp/Ag scaffolds were made, preparing the composite materials with alginate/lidocaine/alginate, lidocaine/alginate/alginate and lidocaine/chitosan/alginate coatings as these coatings showed the best results.

9. Lidocaine hydrochloride delivery systems on the basis of HAp/Ag

FTIR analysis suggested that no chemical reaction between HAp/Ag, lidocaine and alginate/chitosan occurs during the impregnation process. Moreover SEM microphotographs showed that silver particles acted as a crystallization centers for lidocaine crystal growth (see fig.19.).

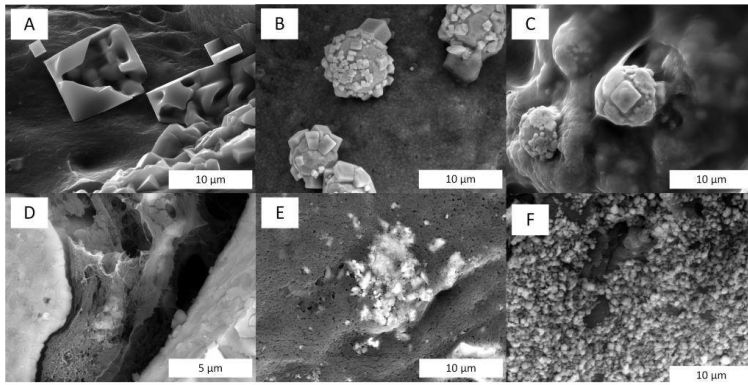


Fig.19. SEM microphotographs: A – HAp/Ag A method alginate/lidocaine/alginate; B – HAp/Ag A method lidocaine/alginate/alginate; C – HAp/Ag A method lidocaine/alginate/chitosan; D – HAp/Ag B method alginate/lidocaine/alginate; E – HAp/Ag B method lidocaine/alginate/alginate; F – HAp/Ag B method lidocaine/alginate/chitosan

From the lidoacaine release results it was concluded that the presence of silver affects the morphology of lidocaine and polymer coating formation; therefore the lidocaine was released faster form silver-containing scaffolds coated with alginate/lidocaine/alginate and lidocaine/chitosan/alginate compared to the pure HAp scaffolds. For HAp/Ag scaffolds with these coatings all introduced lidocaine was released in the first 10 to 24 h. Controlled lidocaine release can be obtained if the HAp/Ag porous scaffolds are impregnated with lidocaine and 2 times coated with alginate (see fig.20.). Controlled drug release on the basis of this material was observed from 25 to 360 h (two weeks).

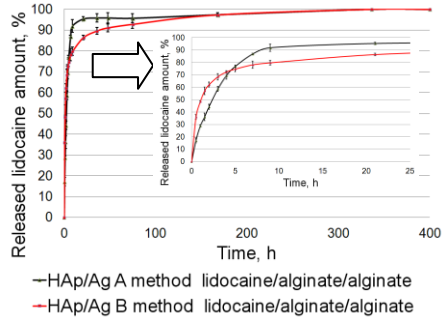


Fig.20. Lidocaine release kinetics from HAp/Ag lidocaine/alginate/alginate composite materials

Lidocaine release rate equilibrium sets later for HAp/Ag A method scaffolds and in the first few hours released lidocaine amount is 3 to 6 % higher compared to HAp/Ag B method scaffolds. It could be related with scaffold pore structure and crystallization of lidocaine on the silver containing particles.

Considering the obtained results and on the basis of literature data additionally HAp/Ag samples were modified with lidocaine and alginate where alginate coating was cross-linked with calcium chloride to obtain the controlled drug release up to 30 days.

The FTIR analysis approved the cross-linking of alginate coating. But as the Ag^+ ions were released from the scaffolds, they linked with COO^- group. Due to that, the increase of symmetric C=O group absorption band was observed and in the case of HAp/Ag B method scaffolds COO^- absorption band intensity was higher, as the Ag^+ ions were released two times faster in the first days compared to HAp/Ag A method scaffolds.

Release kinetics of lidocaine from HAp/Ag lidocaine/alginate+$CaCl_2$ scaffolds did not show the expected results. If compared with the HAp/Ag scaffolds with non cross-linked alginate coating, in the first 10 hours faster lidocaine release was observed (see fig.21).

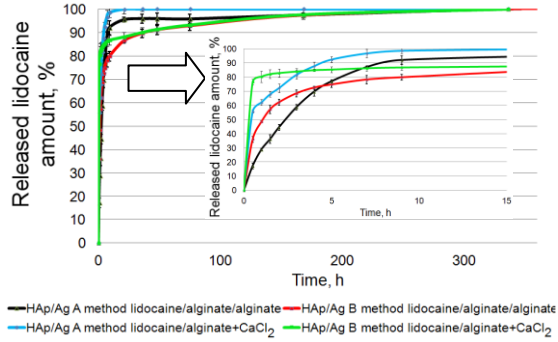


Fig.21. Lidocaine release kinetics from HAp/Ag lidocaine/alginate+$CaCl_2$ scaffolds

Faster drug release can be related to scaffold morphology, cracks, pore structure and silver particles on the surface of pores. All these factors affect the formation of the coating by creating defects in it and as a result faster lidocaine release was observed.

Lidocaine release data showed that the most effective delivery system on the basis of HAp and HAp/Ag scaffolds can be obtained if the scaffolds are impregnated with lidocaine and then two times coated with alginate. Such coating on the HAp/Ag porous scaffolds provides lidocaine delivery up to two weeks.

10. Dexamethasone sodium phosphate delivery systems

Based on the literature research about the dexamethasone (DEXA) immobilization on chitosan scaffolds and layers, this method was transferred to HAp and HAp/Ag ceramic scaffolds.

For HAp and HAp/Ag A method scaffolds FTIR analysis approved the chitosan C=O and C=C (links in the cycle) links conjugation with C=O links in dexamethasone. In the case of HAp/Ag B method scaffolds additional absorption band was not observed, suggesting that DEXA has not linked to the chitosan coating.

Obtained scaffold morphology indicated that depending on the scaffold composition, DEXA crystalline structure and bonding to chitosan can be variable. Changes of scaffolds morphology during the modification process are summarized in figure 22.

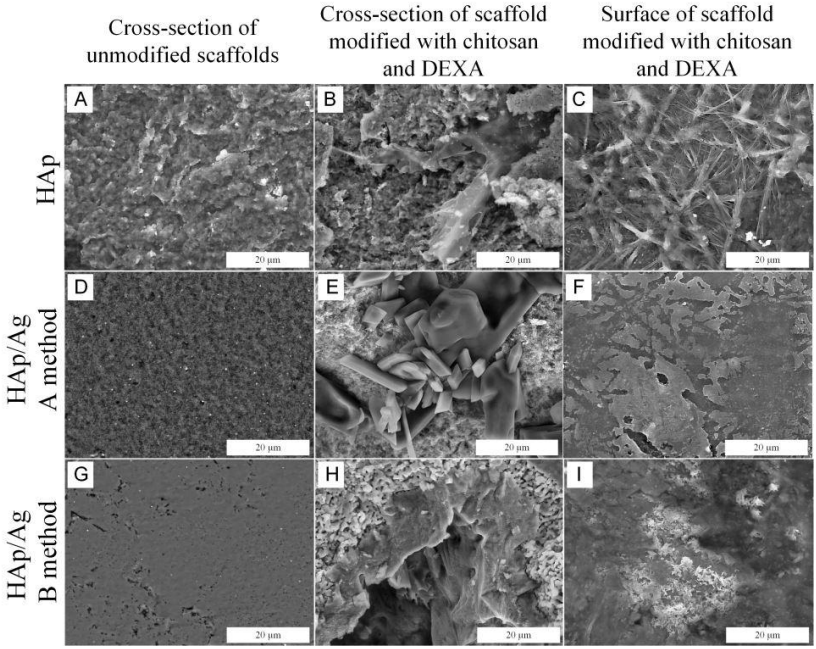


Fig.22. HAp (A – C), A method HAp/Ag (D – F) and B method HAp/Ag (G – I) scaffolds: A, D, G – unmodified; B,E, H – cross-section of scaffolds modified with chitosan and DEXA; C,F,I –surface of scaffolds modified with chitosan and DEXA

DEXA release kinetics was determined in the SBF solution by considering the linked DEXA amount (see fig.23).

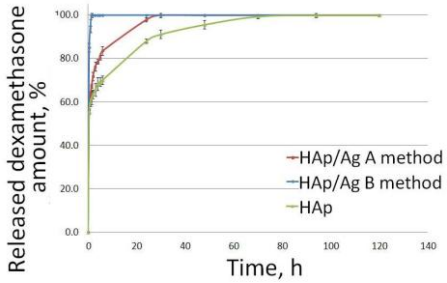


Fig.23. DEXA release kinetics from dense HAp and HAp/Ag scaffolds

DEXA from the dense HAp/Ag B method scaffolds in the SBF solution was released within the 1.5 hours. These results approved bonding between DEXA and chitosan has not occurred. Controlled DEXA release up to 70 h was obtained if the HAp scaffolds were used. In the case of HAp/Ag A method scaffolds, DEXA was released within 30 h. Due to that it can be concluded that release of silver ions form HAp/Ag scaffolds prevents DEXA linkage to the chitosan coating.

If the porous HAp and HAp/Ag scaffolds were modified with chitosan and DEXA, it was possible to delay the DEXA release from A method HAp/Ag scaffolds up to 40 h, from HAp scaffolds up to 70 h and from B method HAp/Ag scaffolds up to 5 hours (see fig.24.).

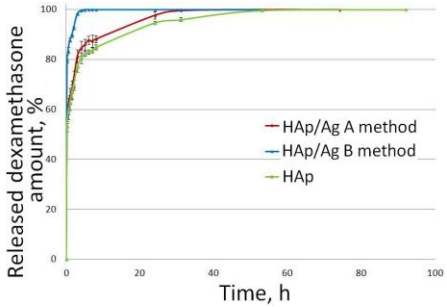


Fig.24. DEXA release kinetics from porous HAp and HAp/Ag scaffolds

DEXA release results from porous HAp and HAp/Ag scaffolds correspond to previous results obtained for dense HAp and HAp/Ag scaffolds. In both cases the fastest DEXA release was observed for B method HAp/Ag scaffolds. Because all amount of DEXA from the B method HAp/Ag scaffolds was released within 5 hours, it can be concluded, that silver ion release from scaffolds prevented the linkage of chitosan and DEXA. DEXA linkage to the chitosan with DIPEA as catalyst is a

nucelophilic reaction and silver cation take part in the reaction; therefore the reaction between DEXA and chitosan did not occur.

The scaffolds were incubated for one month in the SBF solution after the DEXA release tests were done. After a month on the HAp/Ag A method porous scaffolds dense HAp layer was observed (see fig.25.A.), on the HAp/Ag B method scaffolds amorphous HAp layer was observed (see fig.25.B.) indicating that modified HAp/Ag scaffolds are bioactive materials. At the same time on the surface of HAp scaffolds not only new HAp layer was observed but also the formation of bacterial colonies (see fig.25.C. and 25.D.) were detected. In the case of silver-containing materials no bacterial cells were observed in long term SBF experiments, approving the antibacterial activity of scaffolds.

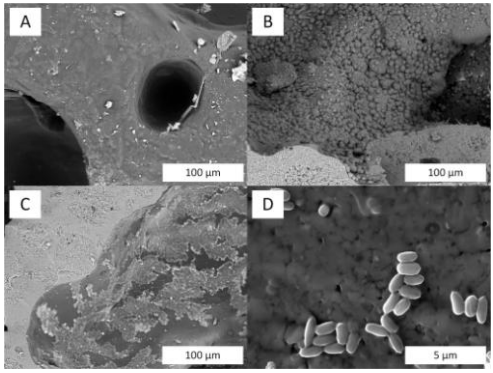


Fig.25. Scaffolds incubated in SBF after DEXA release experiments: A – A method HAp/Ag, B – B method HAp/Ag, C and D: HAp

CONCLUSIONS

1. It is possible to obtain silver doped hydroxyapatite scaffolds with silver content up to 2.51 ± 0.07 wt% if different precursors and preparation technologies are used.
2. Phase composition of silver doped hydroxyapatite and its changes depend on the material preparation method, incorporated silver amount and thermal treatment temperature.
3. The scaffold phase composition and surface area defines the release rate of silver ions from HAp/Ag scaffolds. Silver ions in the therapeutical concentrations (0.025 ± 0.008 μg/L per hour) are released from the prepared scaffolds for more than one year. Silver release rate and released concentration can be controlled using an appropriate HAp/Ag preparation method and formation process.
4. Antibacterial activity of silver doped hydroxyapatite depends on the material thermal treatment temperature as well as on the silver ion release rate and content in the material.
5. It is possible to obtain controlled drug delivery systems where release rate of the active substance depends on the coating and method of the active substance introduction, if the silver doped hydroxyapatite scaffolds are modified with sodium alginate and/or chitosan.
6. If $Ca(NO_3)_2$, $(NH_4)_2HPO_4$, NH_4OH and $AgNO_3$ are used as precursors in the silver doped hydroxyapatite preparation, fast silver ion release in the first days prevents the scaffold linkage between the chitosan and dexamethasone sodium phosphate.
7. Silver particles on the pore surface of silver doped hydroxyapatite porous scaffolds act as crystallization centers for lidocaine hydrochloride.
8. The use of sodium alginate and chitosan coatings for the development of lidocaine hydrochloride delivery systems on the basis of silver doped hydroxyapatite ensures the controlled drug release from 3 up to 15 days.
9. Linkage between chitosan coating and dexamethasone sodium phosphate on the surface of silver doped hydroxyapatite ensures the controlled drug release form 2 to 3 days.

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6. L.Poca, A.Dubnika, D.Loca, L.Berzina-Cimdina. Evaluation of silver doped hydroxyapatite scaffold bioactivity in simulated body fluids. *Key Engineering Materials*. **2014**, 604, 175-179.
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Submitted collective monograph

D.Loca, J.Locs, A.Dubnika, V.Zalite, L.Berzina-Cimdina. 12. Porous hydroxyapatite for drug delivery. Chapter content already submitted in the „Hydroxyapatite (HAP) for biomedical applications" (Edited by: Professor Michael Mucalo, University of Waikato, New Zealand, Woodhead publishing). ISBN – 13:878 1 78242 033 0. Scheduled publication time is the beginning of year **2015**. 36 p.

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4. A.Dubnika, D.Loca, B.J. Nebe, L.Berzina-Cimdina. In vitro studies of osteoblast activity on biphasic calcium phosphate ceramic surfaces. *5th Annual meeting of the Scandinavian Society for Biomaterials*, **2012**, May 8 – 9, Uppsala, Sweden. Abstract published in European Cells and Materials, Volume 23, Supplement 5, 2012, p 34. Poster presentation.
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6. A.Dubnika, I.Salma, D.Loca, L.Berzina-Cimdina. Controlled Release of Dexamethasone from Fibrin Mixed with Biphasic Calcium Phosphate Bioceramics. *RTU 53rd International Scientific Conference*, **2012**, October 11 – 12, Riga, Latvia. Book of abstracts, page No.88. Oral presentation.
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11. A.Dubnika, L.Poca, A.Miglane, D.Loca, V.Rudovica, L.Berzina-Cimdina. Preparation and evaluation of porous silver doped hydroxyapatite scaffolds. Riga Technical University 54th International Scientific Conference. **2013**, October 14 – 16, Riga, Latvia. Book of abstracts, page No. 41. Oral presentation.
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