**RIGA TECHNICAL UNIVERSITY**
Faculty of Materials Science and Applied Chemistry
Institute of General Chemical Engineering

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Student of the Doctoral Study Programme "Materials Science"

# SYNTHESIS OF STRONTIUM AND FLUORINE SUBSTITUTED CALCIUM DEFICIENT HYDROXYAPATITE AND APPLICATION FOR TOOTH ENAMEL REMINERALIZATION

**Summary of the Doctoral Thesis**

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Asociated Professor, *Dr. sc. ing.*,
J. LOČS

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# THE DOCTORAL THESIS PROPOSED TO RIGA TECHNICAL UNIVERSITY FOR THE PROMOTION TO THE SCIENTIFIC DEGREE OF DOCTOR OF ENGINEERING SCIENCES

To be granted the scientific degree of Doctor of Engineering Sciences, the Doctoral Thesis will be defended on 31 May 2017 at the Faculty of Materials Science and Applied Chemistry of Riga Technical University, 3 Paula Valdena Street, Room 272.

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DECLARATION OF ACADEMIC INTEGRITY

I hereby declare that the Doctoral Thesis submitted for the review to Riga Technical University for the promotion to the scientific degree of Doctor of Engineering Sciences is my own and does not contain any unacknowledged material from any source. I confirm that this Thesis has not been submitted to any other university for the promotion to other scientific degree.

Vita Zālīte _____

Date: _____

The Doctoral Thesis has been written in Latvian. It contains an introduction, 3 chapters (Literature Review, Description of Experiments, Results and Discussion), conclusions, references with 168 reference sources and 1 appendice. The volume of the Doctoral Thesis is 135 pages. It has been illustrated by 75 figures and 22 tables.

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# SATURS

# GENERAL OVERVIEW OF THE DOCTORAL THESIS

## Current Solutions and Problems in the Field

The newest tendencies in dentistry indicate that the specialists want to interfere as little as possible in the natural remineralization process of the teeth unless the lesion has penetrated into deeper tissue layers. As the dental specialists dedicate a great deal of attention to monitoring of caries (unless the patient does not keep his or her appointment regular) the amount of research concerned with active substances or remineralization agents and innovative products has risen substantially. It is deemed that these measures have preventive action and they prevent the development of caries in its initial stages. Based on the available information, it must be concluded that the concept of remineralization as such was established in 1960–1970, but more intensive research was started around 2006.

Calcium phosphates, especially hydroxyapatite (HAp), were, are and will be extensively researched as materials for bone tissue substitution and regeneration. Now calcium phosphates have attracted the attention of dental specialists due to their potential in applications concerned with remineralization of tooth enamel. Preparations for oral hygiene which contain HAp are past the clinical research stage and are considered to be effective for prevention of development of caries. As HAp consists of three different functional groups, it is suitable for various chemical modifications to improve or change its chemical, physical and structural properties. Therefore, it is possible that by incorporating into HAp structure such elements as fluorine and strontium the remineralization effect of HAp could be improved.

Scientific literature sources concerned with remineralization *in vitro* were analysed, and it was concluded that individual research projects used very large array of experimental conditions. This makes difficult an objective analysis of results and also drawing conclusions concerning individual remineralization agents. Therefore, there is a necessity for unified body of experimental procedures in such research projects.

## Aims of the Doctoral Thesis

Based on the analysis of the scientific literature and the necessity for effective oral hygiene preparations that are able to provide preventive defence against caries, the following aims were proposed:

- to synthesize bioactive calcium deficient hydroxyapatite (CDHAp), strontium substituted calcium deficient hydroxyapatite (SrCDHAp), fluorine substituted calcium deficient hydroxyapatite (FCDHAp) and strontium and fluorine co-substituted calcium deficient hydroxyapatite (SrFCDHAp) nanoparticles that would as part of oral hygiene preparations facilitate the tooth remineralization process;
- to compile an experimental procedures for research of remineralization process and to conduct experiments to assess remineralization.

## Tasks of the Doctoral Thesis to Complete the Proposed Aims:

- to conduct a review of scientific literature on calcium phosphates (CaP) that exhibit a tooth remineralizing effect and to summarize information about currently available oral hygiene preparations that contain CaP;

- based on the review of scientific literature, to synthesize CDHAp substituted with strontium (up to 10 wt. %) and fluorine (up to 3 wt. %);
- to formulate a composition for a model paste that provides an objective assessment calcium phosphate remineralization potential *in vitro*;
- to prepare and adapt a body of remineralization research methods from the data available in the scientific literature and to describe experimental procedures for evaluation tooth enamel remineralization;
- to conduct remineralization experiments and to assess the remineralization potential of CaP.

**Scientific Significance and Novelty of the Doctoral Thesis**

- CDHAp co-substituted with fluorine and strontium has been synthesized for the first time using a wet precipitation method.
- The influence of model paste containing CDHAp simultaneously substituted with fluorine and strontium has been evaluated for the first time.
- A body of experimental pocedures to characterize tooth enamel remineralization by CDHAp and substituted CDHAp has been described.

**Practical Significance**

A body of experimental pocedures and analytical methods has been developed to evaluate the potential use of CDHAp and CDHAp substituted with Sr and/or F for tooth enamel remineralization.

**Thesis Statements to Be Defended**

1. It is possible to obtain Sr and F co-substituted CDHAp nanoparticles *via* wet precipitation method.
2. Suspension of CDHAp, FCDHAp, SrCDHAp, and SrFCDHAp particles raise pH of acidic aqueous environment.
3. If tooth enamel is treated with model pastes containing CDHAp, FCDHAp, SrCDHAp, and SrFCDHAp particles, the surface of tooth enamel is covered by CaP layer that becomes thicker with increase of treatment cycles.

**Approbation of the Results**

The scientific results of the research within this Doctoral Thesis have been summarised in 12 scientific articles and presented at 12 international conferences.

# LITERATURE REVIEW

Oral health problems that are related to the health of the teeth and solving of these problems are currently very important research topics. Moreover, this research is done not only by practicing dentists but also biologists and materials scientists. Researchers are paying more attention to processes connected with the development of hard tissue of the teeth and to factors that affect remineralization-demineralization equilibrium. Understanding these processes is the basis for innovative ideas and solutions in preventive and therapeutic dentistry.

Tooth enamel is the hardest tissue found in vertebrates. Tooth enamel consists of 95 wt. % of HAp, 4 wt. % of water and 1 wt. % of organic substances (mainly proteins) [1]. The smallest structural units of tooth enamel are needle-shaped, hexagonal HAp nanocrystals. These crystals are placed parallel one to another and gathered into fibres that are 40–60 nm wide and a few hundred micrometers long. These fibres are structures into 4–8 μm wide units that are called enamel rods or enamel prisms [2, 3]. It must be noted that there are no live cells in mature enamel that could repair the lost enamel tissue – unlike it is with bone and dentin. Therefore, the only way for demineralised enamel to be repaired is by binding ions found in saliva ($Ca^{2+}$, $PO_4^{3-}$, $HPO_2^{-}$ and others) to enamel. In this process new crystallization centers are formed that are transformed into HAp crystals. An important factor is the natural buffering capacity of saliva that balances pH changes in the mouth. However, the contemporary bad eating habits and wrong choice of foods tilt the remineralization-demineralization equilibrium towards demineralization for such an amount that natural protective processes and mechanisms cannot renew the lost minerals of enamel. In these cases, oral hygiene preparations that have the maximum capacity for repair of the damaged enamel must be chosen.

In both laboratory and clinical studies during many decades, it has been proven that fluorine-containing formulations, coatings and oral hygiene preparations are effective for strengthening enamel and for prevention of caries. Nevertheless, there are many studies dedicated to the development of new components and materials that contain fluorine ions – as it is not yet ascertained how fluorine ions and enamel interact to strengthen the enamel. There are three main opinions:

a) fluoride ions are included into natural enamel mineral,
b) fluoride ions together with $Ca^{2+}$ ions in saliva form insoluble $CaF_2$,
c) fluoride ions together with $PO_4^{3-}$ and $Ca^{2+}$ ions in saliva form substituted HAp crystals to renew the demineralised enamel.

In its turn, strontium salts have so far been included to decrease tooth sensitivity; this effect has been proven by studies [4]. Nevertheless, there are no studies concerned with strontium and remineralization of hard tissues of teeth. Both $F^-$ and $Sr^{2+}$ ions have been shown to have an effect of metabolism inhibition in bacteria. This indicates that the presence of these ions could lessen the concentration of acids produced by caries-inducing bacteria as this factor most directly affects the progress of demineralization of enamel.

In the last 15 years CaP, mainly in form of amorphous calcium phosphate and HAp, have gained popularity as remineralization agents for hard tissues of teeth. Significant attention is paid to not only the chemical composition but also to particle morphology – for it to be as similar as possible to HAp nanocrystals in enamel or dentine. The advantages of these particles to facilitate the remineraization process are such:

a) they can penetrate nanosized surface defects in the enamel, reaching the lower layers of enamel and even dentin;

b) they have large surface area and this means that the material will be more soluble and reactive – this way the concentration of $Ca^{2+}$ and $PO_4^{3-}$ ions will be increased in the oral cavity.

To synthetically produce particles in this form, wet precipitation method is the most suitable; therefore, this method is also employed in the experimental part of this work.

SrCDHAp and SrFCDHAp have not yet been studied and included in oral hygiene preparations, but there is information available on using FCDHAp and CDHAp to provide and promote remineralization. Therefore, it is necessary to evaluate the remineralization potential of all four components and their possible use in preventive and therapeutic dentistry.

## MATERIALS AND METHODS

The experimental part of this Doctoral Thesis can be divided into two main parts: synthesis of CaP and *in vitro* remineralization studies. Fig. 1 shows the main stages of experimental plan and the instrumental methods used in these stages.

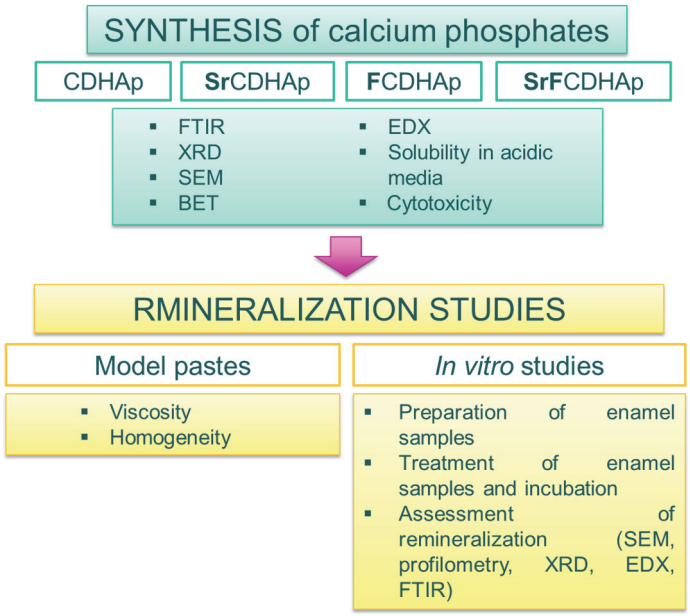


Fig. 1. Main stages of experimental plan.

In the Materials and Methods section of this Doctoral Thesis, the precipitation of CDHAp, FCDHAp, SrCDHAp and SrFCDHAp nanoparticles and the specific synthesis conditions for each product are described. To synthesize SrFCDHAp two alternative synthesis methods were chosen. The methods employed for characterization of obtained products are listed in Fig. 1. To synthesize CDHAp, FCDHAp, SrCDHAp and SrCDHAp nanoparticles, the following reagents were used: $Ca(NO_3)_2$, CaO, $Sr(NO_3)_2$, SrO, $NH_4F$, $(NH_4)_2HPO_4$ and $H_3PO_4$. To ascertain the molar ratio of Ca/P or Ca+Sr/P and the presence of fluorine in the FCDHAp and SrFCDHAp structure, samples of the powders were thermally treated at 1100 °C for 1 hour.

Remineralization studies are divided into two parts: the development of CaP model pastes and *in vitro* studies of tooth enamel remineralization. Before incorporation into model pastes CaP precipitates (pCDHAp) were lyophilized (lyoCDHAp) or spray-dried (spCDHAp) to ascertain if pretreatment of CaP precipitates is needed. To objectively evaluate remineralization effect of the synthesised CaP model pastes

consisted of only 4 components: CaP (5 wt. %, 10 wt. %, 20 wt. %), 2-hydroxycellulose, water and glycerol. The changes in homogeneity of consistency were analyses and viscosity measured for various compositions of model pastes; the model pastes were also compared to commercially available toothpaste.

Bovine tooth enamel was chosen for *in vitro* studies as its structure is the most similar to human enamel according to scientific literature. The preparation of enamel samples includes the separation of soft tissue from teeth, separation of tooth crown from the root, division of teeth in samples (3 mm × 4 mm), fixing the tooth samples in sample holders, polishing and etching of samples using $H_3PO_4$. In the Materials and Methods section of the Doctoral Thesis, experimental conditions are described. Briefly, tooth enamel samples were treated with CaP model pastes two times per 24 hours. Before treatment episode samples were stored in 50 mM citric acid for 3 minutes. Between treatments, the samples were stored in experimental solution in an incubator under dynamic conditions at 37 °C. The duration of remineralization studies was 7 days and nights. The techniques used to study the surface of enamel before and after the remineralization experiment are listed in Fig. 1.

# RESULTS AND DISCUSSION

## 1. Characterization of CaP nanoparticles

XRD, FTIR and EDS instrumental methods were used to characterize chemical and phase composition of synthesised calcium phosphates. Specific surface area (SSA) and particle size was characterized using $N_2$ adsorbtion methods, but the size of the crystallites was ascertained using characteristic (002) peak of HAp in XRD patterns. The obtained data are summarized in Table 1.

Table 1

Characterization of Synthesised CaP

| | FCDHAp | SrCDHAp | SrFCDHAp_N | SrFCDHAp_C | pCDHAp | spCDHAp | lyoCDHAp |
|---|---|---|---|---|---|---|---|
| Ca/P ratio | 1.64 | | | | 1.65 | 1.65 | 1.65 |
| Ca+Sr/P | | 1.64 | 1.64 | 1.64 | | | |
| HAp/β-TCP, wt. % * | 86/14 | 82/12 | 86/14 | 84/16 | 89/11 | 89/11 | 89/11 |
| F, wt. % | 1.6 ± 0.1 | - | 1.9 ± 0.2 | 1.5 ± 0.2 | - | - | - |
| Sr, wt. % | - | 7.4 ± 0.4 | 7.1 ± 0.2 | 7.8 ± 0.3 | - | - | - |
| SSA, $m^2/g$ | 71.1 ± 0.2 | 94.5 ± 1.3 | 140.0 ±1.5 | 78.1 ± 0.4 | 82 ± 0.8 | 92 ± 2.0 | 87 ± 1.1 |
| Particle size $d_{BET}$, nm | 30.0 ± 0.1 | 22.6 ± 0.3 | 15.2 ± 0.2 | 26.5 ± 0.1 | 25.0 ± 0.2 | 23.9 ± 0.5 | 24.1 ± 0.2 |
| Crystallite size $d_{002}$, nm | 30 | 27 | 18 | 27 | 27 | 29 | 30 |

* After thermal treatment at 1100 °C for 1 h.

Absorption bands of [$PO_4$] and [OH] characteristic of apatites were detected in FTIR spectra of all synthesised powders. Additionally, the spectra show also the maximum of [$HPO_4$] and [$CO_3$] functional groups. [$CO_3$] absorption bands show that atmospheric carbon dioxide was solvated in the synthesis environment; thus, it was included in the product structure. In its turn, the presence of [$HPO_4$] shows that the synthesized CaP are calcium deficient. After thermal treatment of CaP powder samples, the spectra lost [$CO_3$] and [$HPO_4$] absorbtion bands, but spectra of FCDHAp, SrFCDHAp_N and SrFCDHAp_C showed extra maximum at 3543–3547 $cm^{-1}$ and 735 $cm^{-1}$; this shows that the structure of samples contains fluorine.

The results of an elemental analysis show that pH cycling method is more suited for an inclusion of strontium atoms into SrFCDHAp structure, but nitrate method is more suited for inclusion of fluorine atoms.

SEM micrographs show that the synthesized particles are characterized by needle-like morphology; this is also shown in the scientific literature [5]. The length of particles is from 100 nm to 300 nm, but the diameter – from 30 nm to 50 nm. Particle dimensions shown by SEM micrographs do not agree with the calculated particle and crystallite sizes (Table 1). This question is answered by TEM micrographs (Fig. 2); the micrographs show that the needle-like particles are realy agglomerates composed of nanosized particles whose sizes agree with sizes shown in Table 1. If SrFCDHAp is synthesised by pH cycling method, crystallite sizes of the product (29 nm ± 3 nm) are larger than crystallite sizes of the product synthesised by nitrate method (19 nm ± 3 nm).

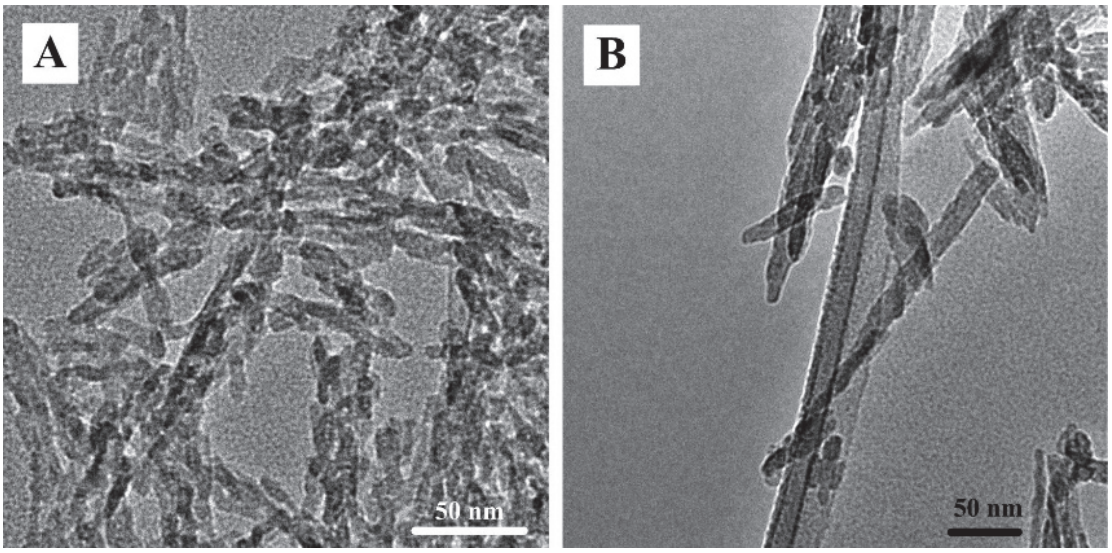


Fig. 2. TEM micrographs: A – SrFCDHAp_N nanoparticles, B – SrFCDHAp_C nanoparticles.

The ability of calcium phosphates to balance the pH of aqueous systems as acid is added is shown in Fig. 3. All samples are characterized by a steep decrease of pH after addition of acid. pH decreases by 1.0–1.2 units in case of synthesized calcium phosphates. In the case of enamel pH decreases by 2.5 units; this means that the critical pH level is reached (pH ≤ 5.5) and the enamel is irreversibly dissolved [6].

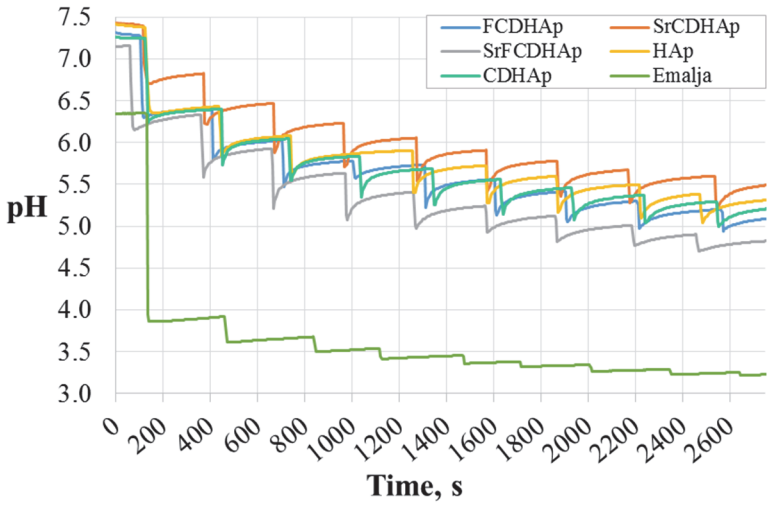


Fig. 3. The dependence in time of pH of CDHAp, FCDHAp, SrCDHAp and SrFCDHAp suspensions after incremental addition of acid.

This experiment shows that, if the enamel should be coated with one of the calcium phosphates, then the coating would be dissolved first and not the enamel. This means that enamel would be protected from erosion caused by acidic foods and from the actions of caries causing bacteria.

The results of cytotoxicity study (Fig. 4) show that, between various concentrations of CaP nanoparticles suspensions, a significant reduction of gingival fibroblast cells (HGF-1 (ATCC® CRL-2014™)) occurs in the case of SrCDHAp and FCDHAp samples, but CDHAp and SrFCDHAp suspensions in various concentrations do not significantly affect the live cell count. If 300 µg/mL concentration samples are compared, a significant reduction of cell count occurred in the case of SrFCDHAp sample – 31 % reduction from the initial cell count. Therefore, it can be concluded that 300 µg/mL SrCDHAp, FCDHAp and CDHAp nanosuspensions do not show cytotoxic effect, but in the case of SrFCDHAp there is a mild cytotoxic effect. *In vitro* experiment show that 500 µg/mL of nanosuspension caused reduction of live cell count by 26 % for SrFCDHAp sample, 37 % for SrCDHAp sample, 42 % for CDHAp and by 63 % for FCDHAp sample. Obtained data show that concentration of 500 µg/mL has mild cytotoxic effect in the case of SrFCDHAp, CDHAp and SrCDHAp nanosuspension samples, but FCDHAp sample shows pronounced cytotoxic effect.

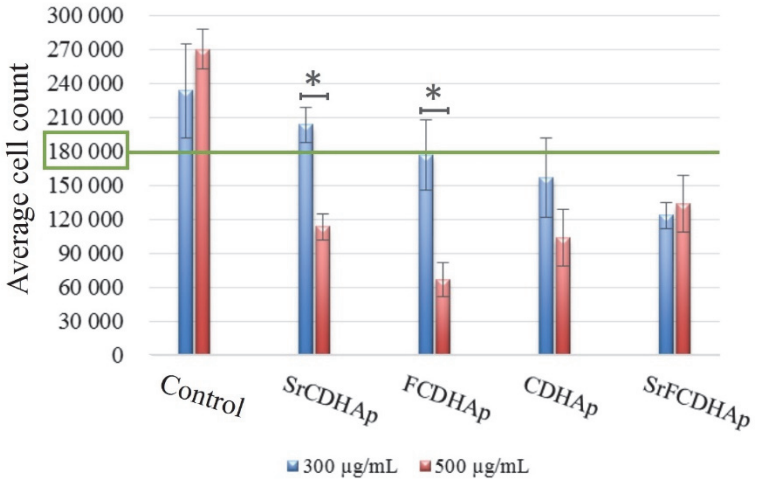


Fig. 4. Live cell count after 48 h exposition to tested nanoparticles suspensions; *$p < 0.05$ – significant difference between data.

## 2. Characterization of Model Pastes

To develop model pastes the influence of morphology of CDHAp particles on preparation process and homogeneity of pastes was evaluated first. It was ascertained that spCDHAp consists of spherical agglomerates of nanosized CDHAp particles around 5 µm in size. In its turn, lyoCDHAp powder was characterized by irregularly shaped agglomerated also consisting of nanoparticles. Untreated CDHAp precipitate does not have such agglomerates as do the two dried powders. Therefore, untreated CDHAp is more easily dispersed in model paste bases so it would contain nanoparticles and not dispersed nanoparticles agglomerates.

Viscosity measurement results for the model pastes are shown in Fig. 5. Viscosity of 5 % and 10 % CDHAp model pastes varies between 2.5 Pa·s and 28.0 Pa·s. Measurements show that the viscosity of pCDHAp_5%, pCDHAp_10%, spCDHAp_5%, lyoCDHAp_5%, lyoCDHAp_10% and lyoCDHAp_20% model pastes does not change with time. This means that the prepared pastes are Newtonian liquids. In their turn, spCDHAp_20%, pCDHAp_20% and *BioRepair* show thixotropic effect as the viscosity under constant shear rate decreases during the whole measurement – and this is an essential property for toothpaste. A viscosity measurements show that the chosen model paste composition with 20 wt. % of CaP meets the standards for rheological properties of commercial toothpastes.

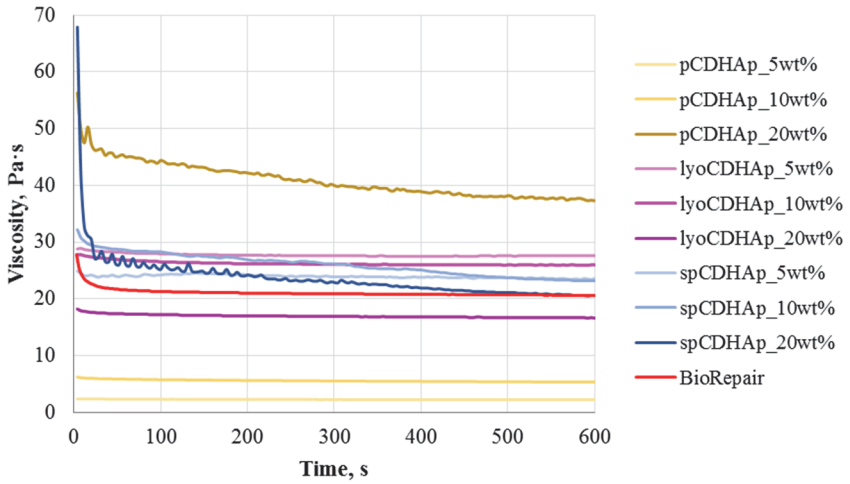


Fig. 5. Dependence of viscosity of prepared toothpaste on time; compared to commercial toothpaste *BioRepair.*

## 3. Remineralization of Tooth Enamel

Etched enamel was characterized using various instrumental methods to evaluate the changes in tooth enamel surface after remineralization studies. Fig. 6 shows XRD pattern of an etched enamel (A) and SEM micrograph (B) of a surface of the enamel. XRD pattern shows that HAp crystals that form the enamel are oriented in a certain way as the pattern does not show all the maxima characteristic of HAp phase. An orientation of HAp crystals is also very well shown by HAp enamel rods in the SEM image. EDS analysis shows that enamel contains the following chemical elements: Ca, P, O, C and N. C and N are found in the organic part of enamel – in proteins.

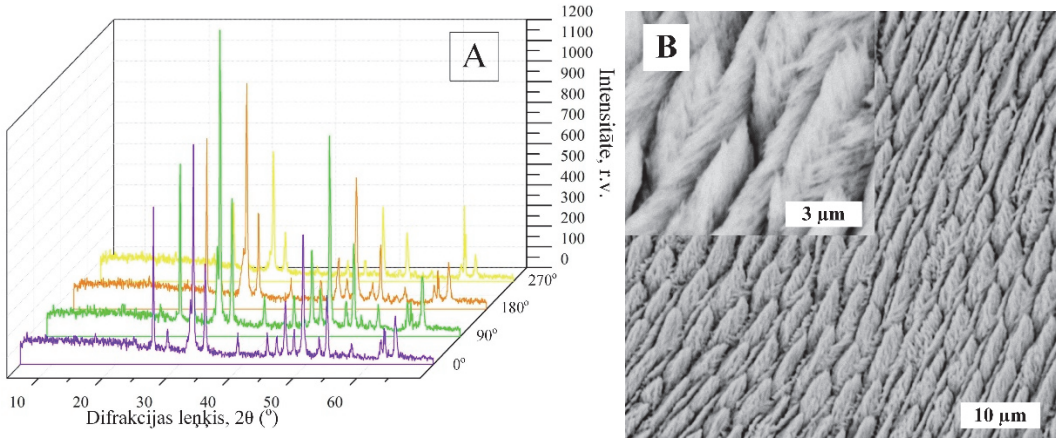


Fig. 6. A – XRD patterns of etched bovine tooth enamel (when XRD patterns were obtained, the orientation of enamel sample was changed by 90° increments against X-ray source, respectively 0°, 90°, 180°, 270°). B – SEM micrograph of etched bovine tooth enamel.

XRD analysis of remineralized enamel samples (Fig. 7) reveals that a new maximum has been generated for all of the samples (for Rem_CDHAp at angle 33.13° 2θ, for Rem_control – 32.89°, for Rem_SrFCDHAp – 32.85°, for Rem_SrCDHAp – 32.91° and for Rem_FCDHAp – 32.93°) when compared to a surface of etched enamel. XRD patterns of Rem_control samples also contain a maximum at 4.8° 2θ; this is characteristic to octa calcium phosphate (OCP) [7].

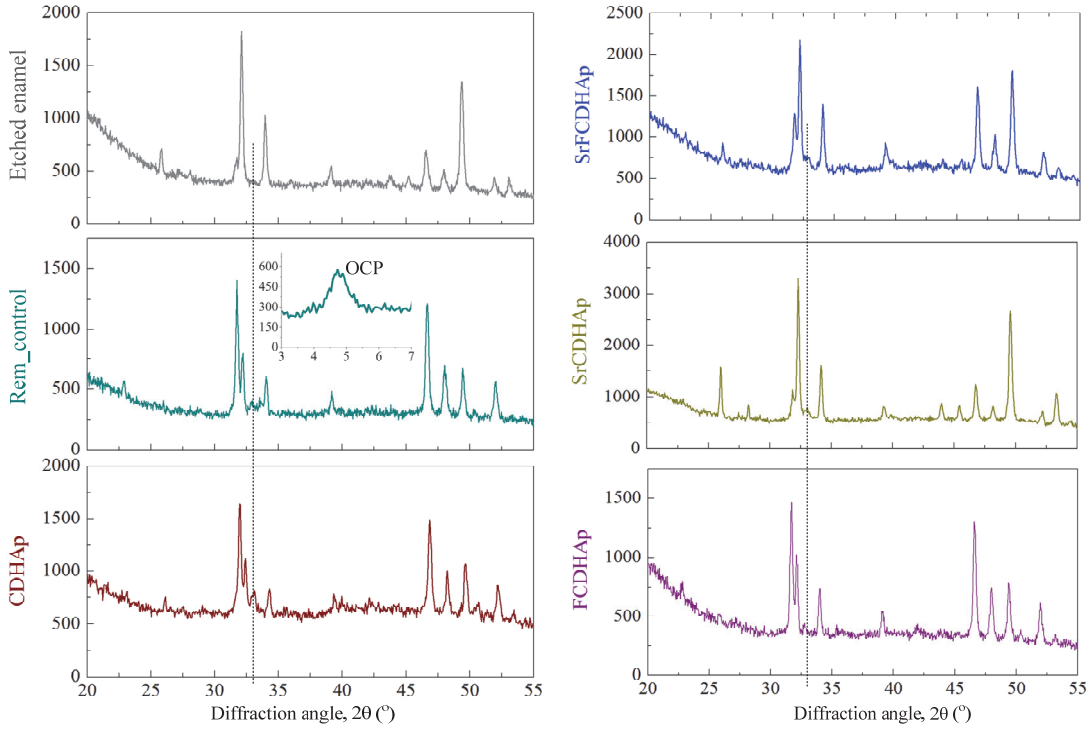


Fig. 7. XRD patterns of enamel surface samples after *in vitro* studies of remineralization (compared to surface of etched enamel); intensity of signals is represented on Y-axis.

Vibrations of absorption bands characteristic of amide groups from 1560 $cm^{-1}$ to 530 $cm^{-1}$ were found in the FTIR spectra of remineralized samples besides absorption bands characteristic of HAp. The absorption bands from 1560 $cm^{-1}$ to 530 $cm^{-1}$ are

characteristic of bending vibration of [N-H] and stretching vibrations of [C-N]. Absorption bands characteristic of [$PO_4$] were detected from 599 $cm^{-1}$ to 472 $cm^{-1}$ for samples starting with etched enamel to SrFCDHAp. Furthermore, in the wide group of maxima from 720 $cm^{-1}$ to 1160 $cm^{-1}$, it is possible that overlap of [$PO_4$] and [$HPO_4$] vibrations have occurred based on the information in the literature; therefore, this means that OCP is present in the samples. For sample Rem_control a small rise is present in this range at 912 $cm^{-1}$ (Fig. 8); this maximum was not observed in other samples. This maximum is characteristic of [P-OH] absorption band of OCP phase according to scientific literature sources [7, 8]. This observation agrees with observations of XRD patterns. The presence of [OH] functional groups is additionally detected as evidenced by a small rise at 630–636 $cm^{-1}$; this is observed in all samples after remineralization experiments with exception of etched enamel sample. The second characteristic absorption band of [OH] vibrations is at ~3565 $cm^{-1}$ (Fig. 8). Also, [$CO_3$] absorption bands at 1410−1450 $cm^{-1}$ were detected in all enamel samples.

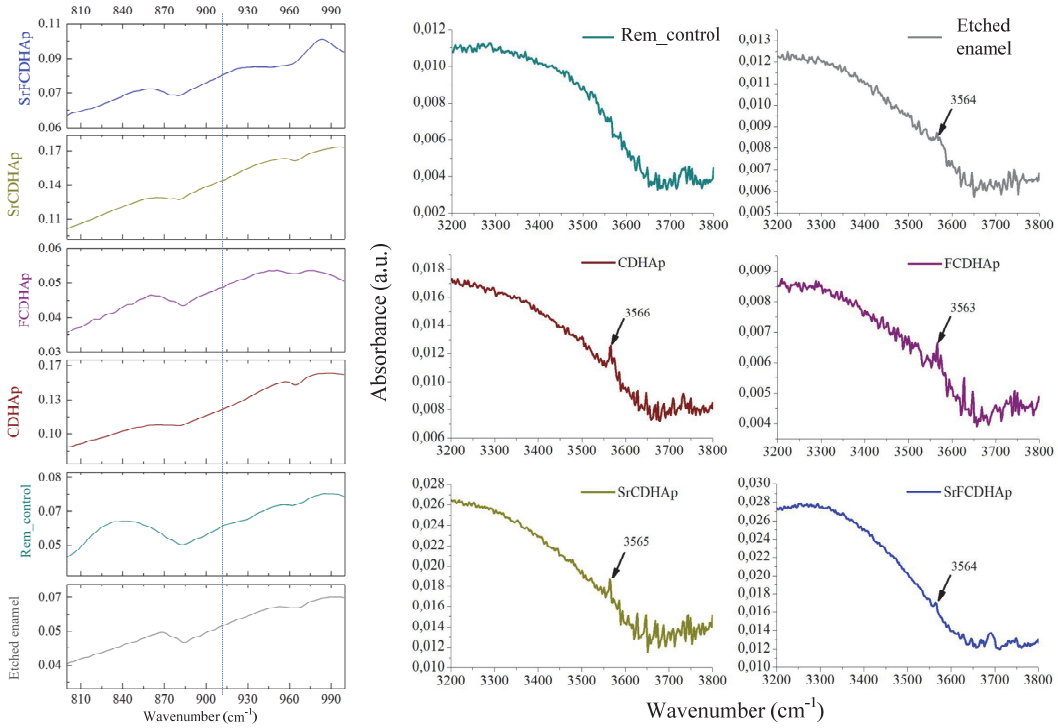


Fig. 8. FTIR spectra of tooth enamel surface after *in vitro* remineralization experiment; absorption intensity in arbitrary units is shown on the Y-axis.

The surface of enamel samples after treatment with spCDHAp_20wt%, lyoCDHAp_20wt% and pCDHAp_20wt% model pastes is shown in Fig. 9. Precipitated spherical particles (some of them are marked using yellow circles) are seen between enamel rods (prisms) in the case of spCDHAp; these particles are characteristic of a spray-dried material. It is essential that the size of these particles is smaller than 2 µm. This may mean that particles with this morphology can be suitable for blocking of open dentinal tubules; this would not only stop the progress of caries but also would give a dental sensitivity lessening effect by blocking the flow of liquid in these tubules that results in irritation of dental nerve [4]. The textures characteristic of etched enamel are not seen after sample was treated with lyoCDHAp; this leads to the conclusion that a calcium phosphate coating has formed. A similar sight can be seen in the case of pCDHAp model paste. As the sample was imaged at higher magnification (D image),

it was found that nanoparticles contained in the composition of pCDHAp model paste have formed a protective coating on the surface of enamel.

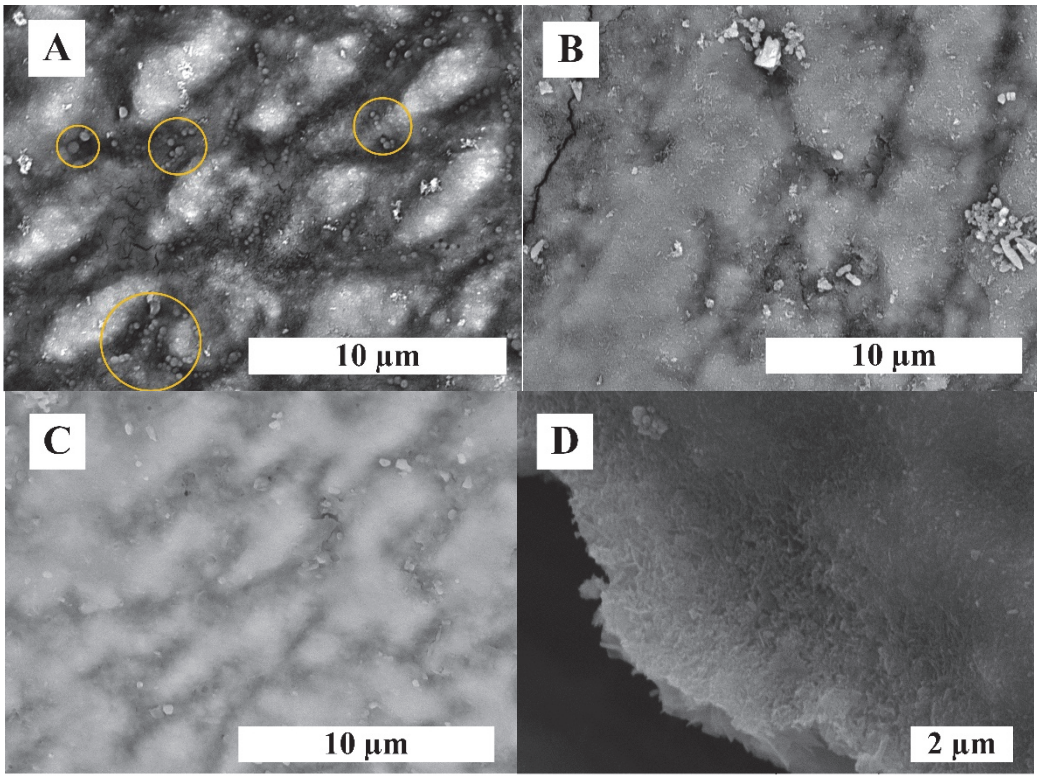


Fig. 9. SEM micrographs after the treatment with spCDHAp_20wt% (A), lyoCDHAp_20wt% (B), pCDHAp_20wt% (C, D) model pastes.

The surface of Rem_control sample is shown in Fig. 10; the border between the original etched surface and the newly formed coating is very obviously shown. The new coating consists of flake-like crystals. Such crystals are characteristic of OCP and HAp [9, 10]. The presence of OCP is also confirmed by FTIR spectra and XRD pattern for the same sample.

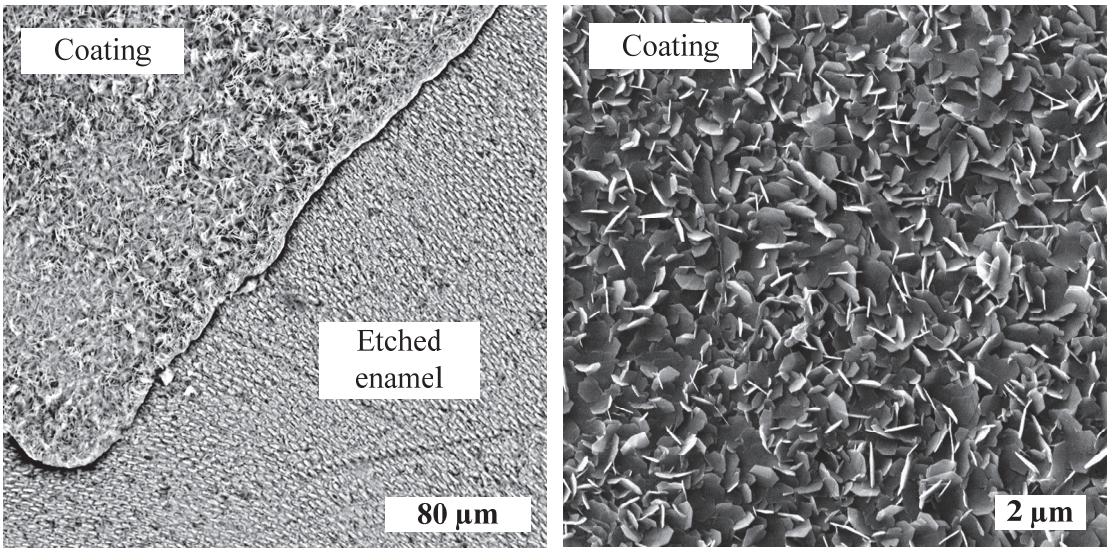


Fig. 10. SEM micrograph of surface of tooth enamel sample Rem_control.

The border between etched enamel surface and the treated surface of Rem_CDHAp sample can also be seen (Fig. 11). But the structure of the protective enamel coating of this sample group is significantly different from the control group.

There are no flake-like crystals, but there are chaotically arranged elongated crystals with rounded ends.

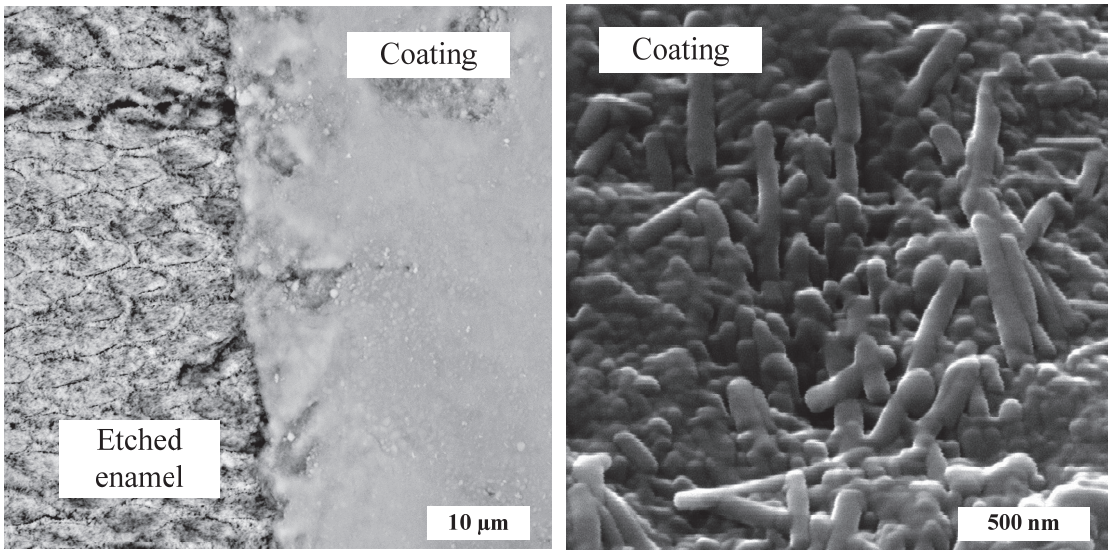


Fig. 11. SEM micrograph of tooth enamel surface for sample Rem_CDHAp.

Fig. 12 shows that the protective coating on Rem_FCDHAp sample group consists of elongated, chaotically placed FCDHAp particles.

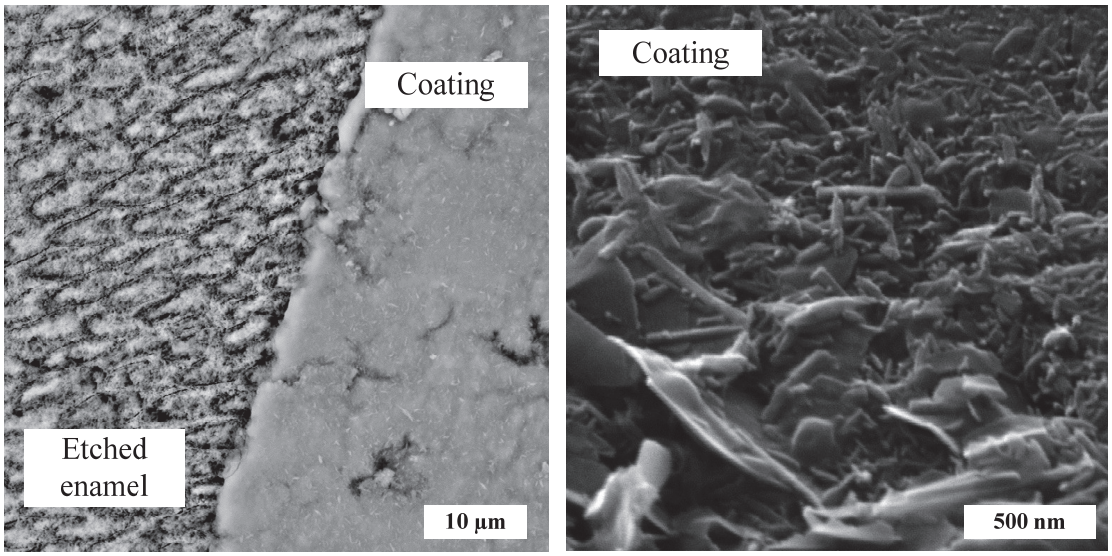


Fig. 12. SEM micrograph of tooth enamel surface for sample Rem_FCDHAp.

SEM micrographs of Rem_SrCDHAp samples (Fig. 13) show tightly packed but unoriented SrCDHAp crystals on the surface of the coating. An interesting observation was that one of these samples had flake-like crystal groups formed in the places where the coating had deepened hollows (Fig. 13 C and D). Similar forms of crystals were observed on samples of a control group. This leads to a conclusion that the newly formed apatite layer is suitable for biomimetic remineralization process to occur that is similar to one occurring on a surface of natural enamel.

Fig. 14 shows on of Rem_SrFCDHAp samples from the sample group. No significant visual differences were observed in the image where a border between the surface of etched enamel and protective coating can be seen when compered to Rem_CDHAp, Rem_FCDHAp, Rem_SrCDHAp.

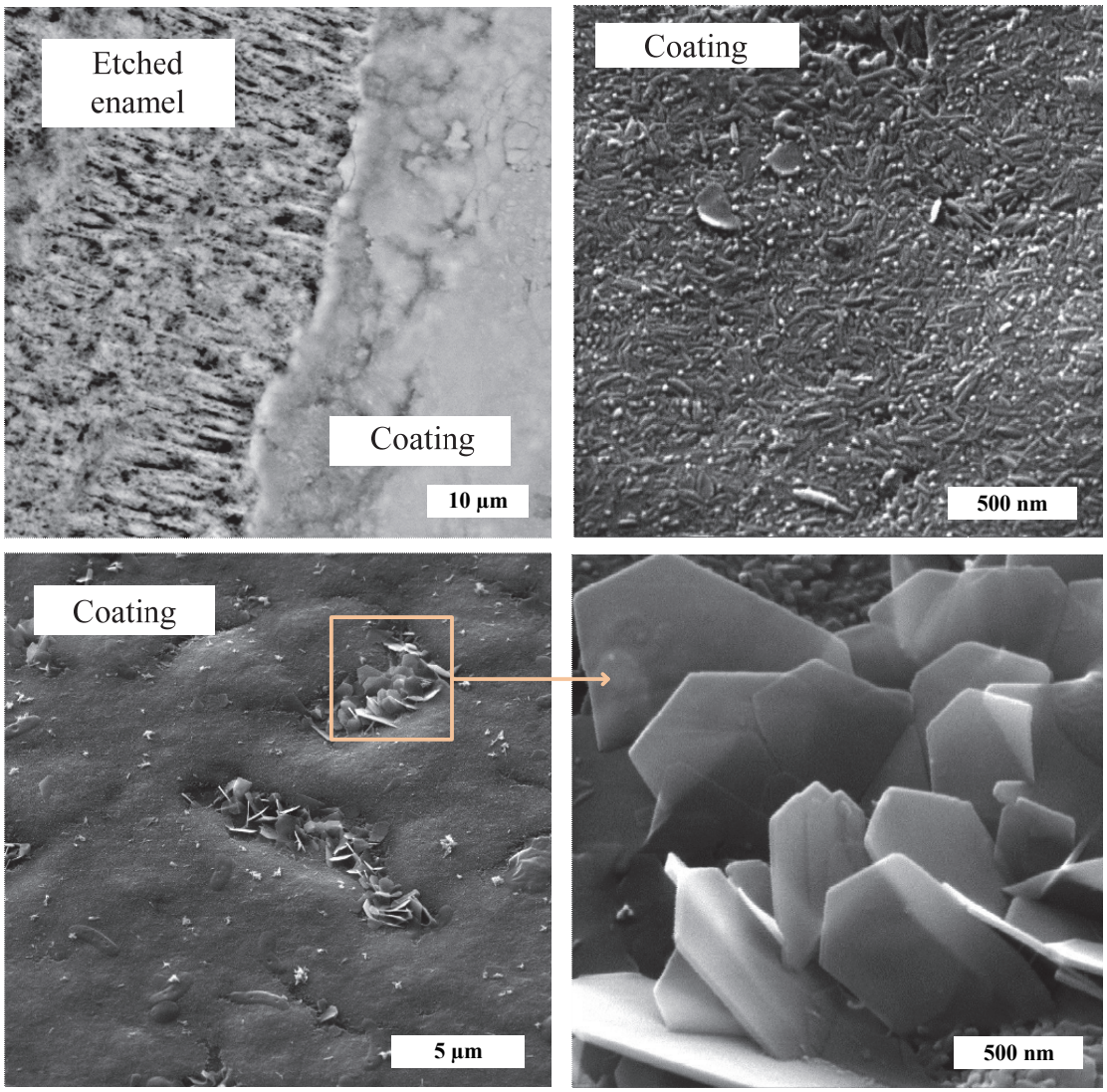

Fig. 13. SEM micrograph of tooth enamel surface for sample Rem_SrCDHAp.

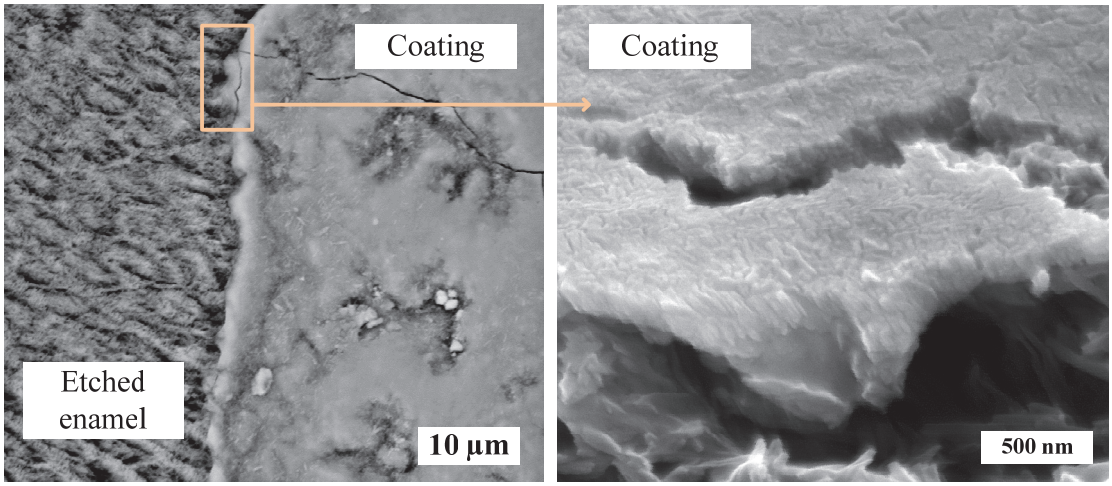

Fig. 14. SEM micrograph of tooth enamel surface for sample Rem_SrFCDHAp.

Nevertheless, when a cracked area of the sample coating was magnified to 100 000 times, it was observed that particles forming it are arranged in parallel to each other and oriented in a certain way. But the particle orientation was not observed in XRD patterns (this would confirm observations in SEM micrographs); therefore, it

would be necessary to conduct extra research using methods suitable for analysis of thin surface layers – for example, using small angle x-ray scattering (SAXS) and SEM that is equipped with focused ion beam (FIB-SEM).

Research of kinetics of formation of protective coating was conducted within the scope of this Doctoral Thesis. The formation kinetics of Rem_CDHAp protective coating during 5 days can be seen in Fig. 15. It was observed that the increase of the thickness of a protective coating was linear (taking into account error margins). The thickness of the protective coating increases by about 0.8 µm ± 0.1 µm in one day. Therefore, it can be concluded that the thickness of protective coating increases as time passes even though enamel samples were subjected to an action of the acid each time before treatment with CaP model pastes.

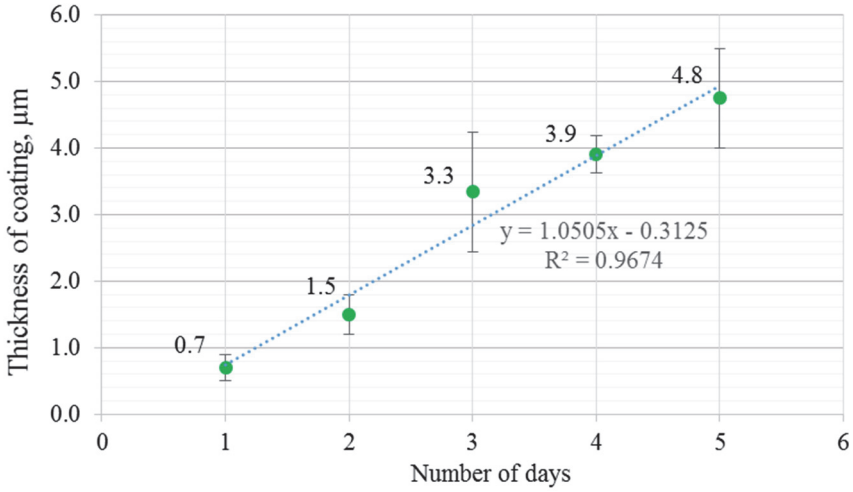


Fig. 15. Kinetics of formation of protective coating on Rem_CDHAp during 5 days.

Both profilogramms and sample surface photosimulations were obtained during profilometry measurements. 3D photosimulations of enamel samples are shown in Fig. 16. Photosimulations of surface Rem_CDHAp, Rem_FCDHAp, Rem_SrCDHAp and Rem_SrFCDHAp enamel samples reveal that border between the protective coating and demineralised enamel surface is step-like. This unambiguously shows that a coating has formed on the sample surface that was not coated with nail polish during the experiments but treated with model pastes instead.

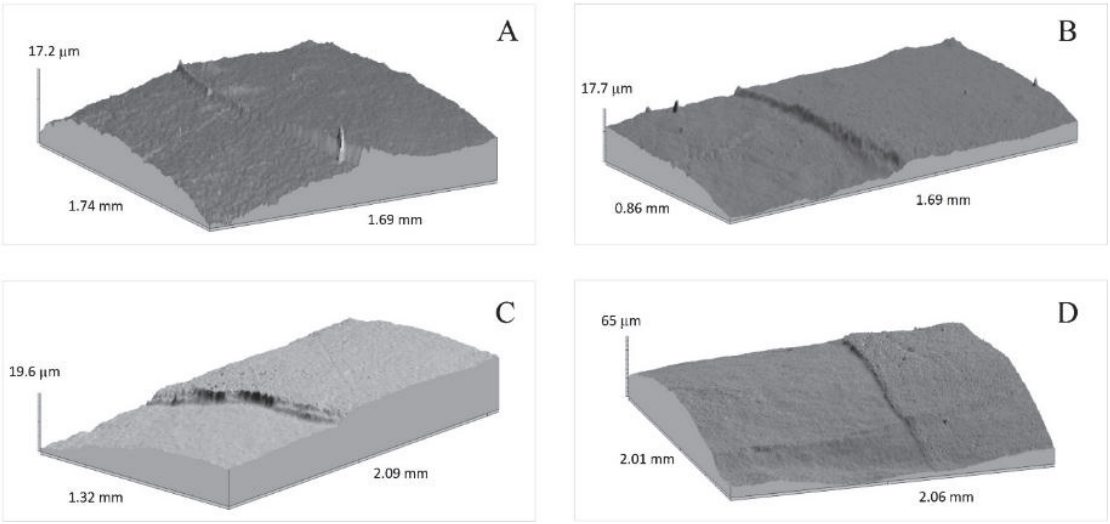


Fig. 16. 3D photosimulations of surfaces of samples of tooth enamel; A – Rem_CDHAp, B – Rem_FCDHAp, C – Rem_SrCDHAp, D – Rem_SrFCDHAp.

The thickness of new CaP coating is characterized by data shown in Fig. 17. The analysis of profilogramms shows that the thickest protective coating has formed on Rem_CDHAp and Rem_FCDHAp samples, but relatively thinner coatings are formed on Rem_SrCDHAp and Rem_SrFCDHAp samples. Nevertheless, analysis of data shows that the differences among various sample groups are not statistically significant. In the research of Thuy *et al.* a solution containing $Sr^{2+}$, $F^{-}$, $Ca^{2+}$ and $H_2PO_3^{-}$ ions was used for remineralization of tooth enamel. After samples were stored in this solution for 14 days, it was ascertained that the presence of both $Sr^{2+}$ and $F^{-}$ ions has promoted the remineralization of damaged enamel surface to the greater extent than the presence of fluoride ions only [11].

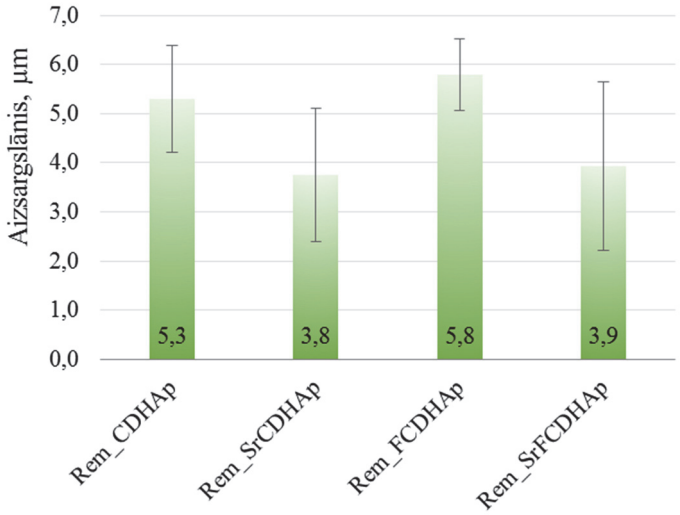


Fig. 17. The thickness remineralized layer on tooth enamel samples after 7-day treatment with Rem_CDHAp, Rem_FCDHAp, Rem_SrCDHAp and Rem_SrFCDHAp model pastes.

It must be noted that it could be important in which form Sr and F ions come into contact with enamel surface – that is – in form of a free ion or already bound in some structure (as it is done within this Doctoral Thesis). Cao *et al.* have researched the effect of a combination of $Ca^{2+}$ ions and enamel matrix derivatives on the remineralization process; it was observed that after 96 h a 3.5 µm thick remineralized layer has formed [12]. Analysis of works by Thuy *et al.* and Cao *et al.* did not lead to unambiguous conclusions because there were different experimental procedures and the materials employed for remineralization procedures were different. After analysis of scientific literature, it was concluded that in order to be able to compare results and to assert statements based on research *in vitro* remineralization procedures must be the same in various experiments.

As humans lose 10–40 µm of layer from the surface of enamel each year because of erosion [4], the inclusion of CaP particles studied in this Doctoral Thesis in oral hygiene preparations (mainly toothpastes) would not only prevent the development of caries but also reduce the erosion of enamel because the protective layer would dissolve and erode first.

Profilometry results supplement the information shown by SEM micrographs (section 3.3.4) and indicate that *in vitro* remineralization experiments lead to significant surface changes; therefore, it can be stated that underemployed conditions of experiments protective coating has formed on all enamel samples treated with model pastes.

# CONCLUSIONS

1. The wet precipitation method is suitable for synthesis of Sr and F co-substituted calcium deficient hydroxyapatite. Moreover, this technique ensures crystallization of needle-like CDHAp nanoparticles similar to HAp found in the mineral part of natural tooth enamel.
2. pH cycling method led to higher $Sr^{2+}$ substitution level and development of larger particles (29 nm ± 3 nm), while nitrate route was favourable for incorporation of $F^-$ ions into CDHAp structure and formation of smaller crystallites (19 nm ± 3 nm).
3. All synthesized CaP exhibit pH buffering effect of an aqueous system after incremental acid addition.
4. The composition of developed model pastes with a viscosity similar to commercial toothpaste ensures a formation of the coating of calcium phosphates on the etched enamel samples.
5. The coating of calcium phosphates was formed on the enamel samples treated with CDHAp, FCDHAp, SrCDHAp and SrFCDHAp model pastes, but on the control enamel samples biomimetic CaP coating, consisting of OCP and HAp phases was detected.
6. The protocol of *in vitro* remineralization of tooth enamel is described. It allows evaluating the potential protective effect of synthesized CDHAp, FCDHAp, SrCDHAp and SrFCDHAp nanoparticles. During remineralization experiment 5.8 μm ± 0.7 μm thick coating was developed in 7 days.

# REFERENCES


[1] M. Epple, E. Baeuerlein, eds., Handbook of Biomineralization. Medical and Clinical Aspects, WileVCH Verlag GmbH & Co, 2007.

[2] P. Vallutti, ed., Non-metallic biomaterials for tooth repair and repalcement, VWoodhead Publishing, 2013.

[3] M. Hannig, C. Hannig, Nanomaterials in preventive dentistry., Nat. Nanotechnol. 5 (2010) 565–9. doi:10.1038/nnano.2010.83.

[4] M. Mantzourani, D. Sharma, Dentine sensitivity: past, present and future., J. Dent. 41 Suppl 4 (2013) S3-17. doi:10.1016/S0300-5712(13)70002-2.

[5] M. Sadat-Shojai, M.T. Khorasani, E. Dinpanah-Khoshdargi, A. Jamshidi, Synthesis methods for nanosized hydroxyapatite with diverse structures, Acta Biomater. 9 (2013) 7591–7621. doi:10.1016/j.actbio.2013.04.012.

[6] S. V. Dorozhkin, Calcium orthophosphates in dentistry, J. Mater. Sci. Mater. Med. 24 (2013) 1335–1363. doi:10.1007/s10856-013-4898-1.

[7] I.A. Karampas, C.G. Kontoyannis, Characterization of calcium phosphates mixtures, Vib. Spectrosc. 64 (2013) 126–133. doi:10.1016/j.vibspec.2012.11.003.

[8] B.O. Fowler, M. Markovic, W.E. Brown, Octacalcium Phosphate .3. Infrared and Raman Vibrational-Spectra, Chem. Mater. 5 (1993) 1417–1423. doi:10.1021/cm00034a009.

[9] J.P. Simmer, a G. Fincham, Molecular mechanisms of dental enamel formation., Crit. Rev. Oral Biol. Med. 6 (1995) 84–108. doi:10.1177/10454411950060020701.

[10] D.O. Costa, B. a Allo, R. Klassen, J.L. Hutter, S.J. Dixon, A.S. Rizkalla, Control of surface topography in biomimetic calcium phosphate coatings., Langmuir. 28 (2012) 3871–80. doi:10.1021/la203224a.

[11] T.T. Thuy, H. Nakagaki, K. Kato, P.A. Hung, J. Inukai, S. Tsuboi, H. Nakagaki, M.N. Hirose, S. Igarashi, C. Robinson, Effect of strontium in combination with fluoride on enamel remineralization in vitro., Arch. Oral Biol. 53 (2008) 1017–22. doi:10.1016/j.archoralbio.2008.06.005.

[12] C. Robinson, S. Connell, S.J. Brookes, J. Kirkham, R.C. Shore, D. a M. Smith, Surface chemistry of enamel apatite during maturation in relation to pH: Implications for protein removal and crystal growth, Arch. Oral Biol. 50 (2005) 267–270. doi:10.1016/j.archoralbio.2004.11.017.

# LIST OF PUBLICATIONS

1. V.Zalite, J.Rimsa, J.Locs, K.Kundzins. Synthesis of strontium and fluorine co-substituted calcium deficient hydroxyapatite nanoparticles for tooth enamel remineralization: two alternative wet precipitation routes. *Journal of materials Chemistry B*, **2017**, (submitted, cited by SCOPUS database)
2. V.Zalite, J.Locs. Characterization and Preparation of Calcium Phosphate Model Toothpaste for Tooth Enamel Remineralization. *Key Engineering Materials,* **2017**, 721, 213–218. (cited by SCOPUS database)
3. G.Salms, V.Ananjevs, V.Kasyanovs, A. Skagers, I. Salma, J.Vetra, V.Zalite, L.Stipniece, S.Petronis. Change of Biomechanical Parameters in the Lower Jaws of Rabbits with Experimental Osteoporosis after Implantation of Calcium-Phosphate Bioceramics Material in the Geater Trochanter Region. *Key Engineering Materials,* **2017**, 721, 224–228. (cited by SCOPUS database)
4. S.Petronis, J.Locs, V.Zalite, M.Pilmane, A.Skagers, I.Salma, G.Salms. Impact of Biphasic Calcium Phosphate Bioceramics on Osteoporotic Hip Bone Mineralization *in vivo* Six Months after Implantation. *Key Engineering Materials,* **2017**, 721, 229–233. (cited by SCOPUS database)
5. J.Rimša, V.Zālīte, J.Ločs. Synthesis of Strontium and Fluorine Co-Substitued Calcium Phosphate. *Riga Technical University 57th International Scientific Conference "Materials Science and Applied Chemistry" (MSAC 2016): Proceedings and Programme,* **2016**, 147–152. (Open Access)
6. V.Zalite, J.Locs. Characterization of different hydroxyapatite particles for tooth enamel remineralization. *Key Engineering Materials,* **2016**, 674, 139–144. (cited by SCOPUS database)
7. V.Zalite, K.Kostrjukova, J.Locs. Preparation of F-doped Hydroxyapatite via Wet-Chemical Precipitation Technique Combined with pH-cycling. *Advanced Materials Research,* **2015**, 1117, 205–209 (cited by EBSCO Host database)
8. A.Dubnika, V.Zalite. Preparation and characterization of porous Ag doped hydroxyapatite bioceramic scaffolds. *Cer. Inter.*, **2014**, 40, 9923–9930 (cited by SCOPUS database)
9. V.Zalite, M.Sokolova, D.Jakovlevs, K.Rozenbergs, L.Berzina-Cimdina. Characterization of rabbit mesenchymal cell attachment on calcium phosphate surface. *RTU scientific articles «Material Science and Applied Chemistry»,* 30th volume, **2014**, pp. 22–27.
10. J.Locs, V.Zalite, L.Berzina-Cimdina. Ammonium hydrogen carbonate provided viscous mass foaming – a novel technology for the preparation of porous ceramics. *J. Eur. Cer. Soc.*, **2013**, 33, 3437–3443. (cited by SCOPUS database)
11. V.Zalite, V.Groma, D.Jakovlevs, J.Locs, G.Salms. Characterization of rabbit mesenchymal stem cell attachment on calcium phosphate surface. *IFMBE Proceedings* 38, **2013**, 194–197 (cited by SCOPUS database)
12. S.Petronis, J.Petronis, V.Zalite, J.Locs, A.Skagers, M.Pilmane. New biphasic calcium phosphate in orthopedic surgery: First clinical results. *IFMBE Proceedings* 38, **2013**, 174–177 (cited by SCOPUS database)

**Submitted collective monograph**

D.Loca, J.Locs, A.Dubnika, V.Zalite, L.Berzina-Cimdina. Section: 9. Porous hydroxyapatite for drug delivery. Redaktors M. Mucalo. Hydroxyapatite (HAP) for biomedical application. ISBN 978-1-78242-033-0, *Woodhead Publishing Ltd*, **2015**, pp. 189–205.

**Patents**

1. WO 2016/178174 A1 "Method for manufacturing bone implants and bone implant" Wojciech Swieszkowski, Tadeusz Chudoba, Sylwia Kusnieruk, Aleksandra Kedzierska, Bartosz Wozniak, Julia Rogowska-Tylman, Dariusz Smolen, Elzbieta Pietrzykowska, Witold Lojkowski, Jacek Wojnarowicz, Aharon Gedanken, Jānis Ločs, Vita Zālīte, Māra Pilmane, Ilze Šalma (published 10.11.2016) .
2. LV 15060 B "Līdzeklis kaulaudu defektu aizvietošanai osteoporozes gadījumā" Andrejs Skaģers, Ilze Šalma, Ģirts Šalms, Jānis Vētra, Sandris Petronis, Māra Pilmane, Jānis Ločs, Vita Zālīte (published 20.04.2016) .

**Participation in the international and local scientific conferences with reviewed conference proceedings:**

1. V. Zalite, J. Locs, J. Lungevics. Strontium and fluorine co-substituted hydroxyapatite for tooth enamel remineralization. 1*0th annual meeting of Scandinavian Society for Biomaterials*, Hafjell, Norway, March 15-17, 2017. *European Cells and Materials* 33:1 (Suppl), p 42, 2017.
2. V.Zalite, J.Locs. Characterization and Preparation of Calcium Phosphate Model Toothpaste for Tooth Enamel Remineralization. *25th International Baltic Conference ENGINEERING MATERIALS & TRIBOLOGY BALTMATTRIB* 2016, Riga, Latvia, November 3–4. Abstact Book p.62.
3. V.Zalite, J.Locs, J. Vecstaudza "The design of calcium phosphate particles for tooth hard tissue remineralization" *15th Euro Congress on Dental & Oral Health* , Rome, Italy, October 24–26, 2016. *Dentistry* 6:9 (Suppl) p.73, 2016.
4. L. Stipniece, J. Vecstaudza, V. Zalite, K. Salama-Ancane, D. Loca, L.Berzina-Cimdina „Optimization of the synthesis and design of calcium phosphates for biomedical application" *LU Cietvielu fizikas institūta 32. zinātniskā konference (LU 74. zinātniskās konferences ietvaros)*, 17.02.–19.02. 2016, Riga, Latvia.
5. V. Zalite, J. Locs „Characterization of different hydroxyapatite particles for tooth enamel remineralization" *24th International Baltic Conference ENGINEERING MATERIALS & TRIBOLOGY BALTMATTRIB*, Tallinn, Estonia, November 5–6, 2015. Abstract Book.
6. V. Zalite, J. Locs „Preparation of substituted hydroxyapatite for tooth enamel remineralization" *27th European Conference on Biomaterials*, Krakow, Poland, August 30 – September 3, 2015. Abstract Book, p.785.
7. V. Zalite, J. Locs „Preparation of Sr and F co-substituted hydroxyapatite via wet-chemical precipitation technique", *8th Conference of Scancinavian Society for Biomaterials*, Sigulda, Latvia, May 6-8, 2015. Abstract Book, p. 93.
8. V.Zalite, J. Locs, K. Kostrjukova "Characterization of fluorhydroxyapatite produced by pH-cycling" *Riga Technical University 55th International Scientific Conference,* Riga, Latvia, October 14–17, 2014.

9. V. Zalite, K. Kostrjukova, J.Locs "Preparation of F-doped Hydroxyapatite via Wet-Chemical Precipitation Technique Combined with pH-cycling" *13th International Conference on Global Research and Education, InterAcademia*, Riga, Latvia, September 10–12, 2014.
10. J. Locs, V. Zalite "Ammonium hydrogen carbonate provided viscous slurry foaming of calcium phosphates for preparation of porous ceramics" *Biointerfaces in Biomaterials*, Aarhus, Denmark, March 26–28, 2014.
11. V. Zalite, D. Jakovlevs, J. Locs "Characterization of rabbit mesenchymal stem cell attachment on calcium phosphate surface" *Biointerface*, Dublin, Ireland, October 23–25, 2012.
12. V. Zalite, "Development of calcium phosphate ceramics in Rudolfs Cimdins Riga Biomaterials Innovations and Development Centre" *Symposium "Biocermics and Cells for reinforcement of bone"*, Riga, Latvia, October 19–20, 2012. Abstract Book, p. 42
13. V. Zalite, V. Groma, D. Jakovļevs, J. Locs, G. Salms "Ultrastructural characteristics of tissue response after implantation of calcium phosphate ceramics in the mandible of rabbit" *International Symposium on Biomedical Engineering and Medical Physics*, Riga, Latvia, October 10–12, 2012.