

# THE CALCIUM PHOSPHATE FORMATION ON TI ALLOY BY PRECALCIFICATION PROCESS UNDER STATIC CONDITIONS

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*The aim of this paper was to study the influence of precalcification process on the nucleation as well as growth of calcium-phosphates crystals (Ca-P) on the surface of Ti alloy. Ti alloy was precalcified by the calcification solutions (SCS1-3) with different content of  $\text{Ca}^{2+}$  and  $(\text{PO}_4)^{3-}$  ions and compared with the non-precalcified sample. The results of  $\text{Ca}^{2+}$  and  $(\text{PO}_4)^{3-}$  analysis indicate that precalcification step accelerates the consumption of both  $\text{Ca}^{2+}$  and  $(\text{PO}_4)^{3-}$  ions from simulated body fluid (SBF) solution. The deposition of Ca-P phase was completed approximately 6 days earlier when compared it with the non-treated samples. Analytical measurements as well as the surface observation enable to find optimal calcification solution marked as SCS2 containing ( $\text{mmol/dm}^3$ ) 4.0  $\text{Na}^+$ , 5.0  $\text{Ca}^{2+}$ , 10.0  $\text{Cl}^-$ , 2.5  $\text{H}_2\text{PO}_4^-$  and 1.5  $\text{HCO}_3^-$ . The influence of S/V ratio (surface area / volume of soaking media) changes on the nucleation and thickness of Ca-P layer formed in the SCS2 solution has been studied in the second part of this work. It was found out that 4 days immersion in SCS2 solution at S/V ratios at 0.1 and 0.5  $\text{cm}^{-1}$ , respectively, is enough time for complete deposition by Ca-P phase on the alloy surface. The changes of the S/V ratio allow adjusting the thickness of Ca-P layer on the surface.*

## INTRODUCTION

Materials based on calcium-phosphate, such as hydroxyapatite, have been shown to enhance bone apposition to orthopedic implants; they do not form fibrous tissues, but instead an extremely thin, epitaxial bonding layer with existing bone. These materials have an excellent bioactive behavior but due to their low mechanical properties they are mostly used as coatings on implant surfaces of substrates such as Ti6Al4V and other medical alloys [1]. Biomimetic coating processes overcome many of the shortcomings of conventional vapor phase coating techniques and are designed to mimic biomineralization processes based on using of the calcification solutions [2-4]. Uniform coatings can be applied to any surface that has access to an aqueous solution. The process involves controlled crystal nucleation and growth. All surfaces within a porous implant can be uniformly coated without dogging or filling the implant.

Titanium materials have been used successfully in orthopedic and dental surgery due to their good mechanical properties for many years. However, in order to successful bonding to hard tissue the Ti alloy surface has to be treated before applying by strong acid and base (HF or HCl and NaOH) at the first step. After this chemical treatment a thin  $\text{TiO}_2$  gel-like layer with presence of  $\text{Na}^+$  ion is formed on the alloy surface. Presence of this layer gives bioactive properties to Ti [5-6]. Chemical analysis of the supersaturated solutions with

the time of exposure of tested materials confirmed continuous but very slow consumption of  $\text{Ca}^{2+}$  as well as  $(\text{PO}_4)^{3-}$  ions [7]. The objective of this work was to choice the more advantageous supersaturated calcification solutions (SCS1-3) and the second part of work was focused on calcium-phosphate growth in SCS2 solution depending on S/V ratio (surface area / volume of soaking media). The quality of calcium-phosphate layer depends on the concentration and ratio of both  $\text{Ca}^{2+}$  and  $(\text{PO}_4)^{3-}$  as well as the presence of  $\text{NaHCO}_3$  in the role of buffer in SCS. Subsequent in vitro testing in SBF indicated an acceleration of new calcium-phosphate forming (probably hydroxyapatite).

## EXPERIMENTAL

*Chemical treatment:* The Ti6Al4V alloy surfaces with the dimension of  $10 \times 10 \times 0.7$  mm (figure 1) were etched in HF (1:10) for 1 min, 5-times washed by demineralized water. Pre-treatment continued by soaking in 10M NaOH solution at  $60^\circ\text{C}$  for 24 hours with subsequent (5-times) gentle rinsing by demineralized water (figure 2).

*Precalcification:* The samples were precalcified in supersaturated calcification solutions (SCS1-3) (table 1). The Ti alloy samples were soaked in SCS solutions under static condition at  $37^\circ\text{C}$  for 7 days at  $S/V = 0.5 \text{ cm}^{-1}$  ( $S$  = sample surface ( $\text{cm}^2$ ),  $V$  = volume of soaking solution ( $\text{cm}^3$ )).

Table 1. The ion concentration of the SCS solutions (mmol/dm<sup>3</sup>).

solution	Na <sup>+</sup>	Ca <sup>2+</sup>	Cl <sup>-</sup>	H <sub>2</sub> PO <sub>4</sub> <sup>-</sup>	HCO <sub>3</sub> <sup>-</sup>
SCS1	6.5	5.0	10.0	5.0	1.5
SCS2	4.0	5.0	10.0	2.5	1.5
SCS3	4.0	10.0	20.0	2.5	1.5

*Exposition in SBF:* The effect of Ti alloy pre-treatment and precalcification on calcium phosphate formation was examined in simulated body fluid (SBF) which simulates inorganic part of human blood plasma (table 2). The SBF solution was prepared by dissolving reagent grade KCl, NaCl, NaHCO<sub>3</sub>, MgSO<sub>4</sub>, CaCl<sub>2</sub> a KH<sub>2</sub>PO<sub>4</sub> into demineralized water and buffered at *pH* = 7.3 with TRIS [tris(hydroxymethyl)aminomethan] and HCl at 37°C. The samples were exposed into SBF solution under static conditions in a biological thermostat at 37°C. The sample without precalcification was soaked in SBF at *S/V* = 0.5 cm<sup>-1</sup> for 14-20 days and precalcified ones at *S/V* = 0.5 cm<sup>-1</sup> for 7-14 days.

*Analytical measurement:* Sample-solution interactions were quantified on the basis of solution analysis. To evaluate the ability and rate of Ca-P formation, the concentration of phosphates and calcium ions in the solution were performed by spectrophotometric measurement and atomic absorption spectroscopy (AAS), respectively.

The analysis of Ca<sup>2+</sup> ions concentration were performed at  $\lambda = 442$  nm by AAS. The KCl releasing buffer with concentration of 4000 ppm was added to each sam-

ple. The (PO<sub>4</sub>)<sup>3-</sup> analyses were performed by spectrophotometric method. The analysis was based on determination of phosphate ions on the yellow form at 460 nm. Reproducibility of results was 5-10 %. Error bars in time dependences represent maximum difference for 2 independent measurements.

*pH measurement:* Value of *pH* was measured at 25°C by glass electrode.

*Analysis of sample surface:* Ti alloy surfaces after chemical treatment and exposure in precalcification and subsequently in SBF solution were observed by scanning electron microscope Hitachi S4700 (SEM-EDS) and by optical microscopy using image analysis with software LUCIA. The thickness of the calcium-phosphate layers was observed on the cross section of the samples fixed in resin and subsequently polished by 3  $\mu$ m grains of diamond paste. WAXS-GI XRD methods confirmed crystal character of newly formed layers.

## RESULTS AND DISCUSSION

Figure 2 shows the morphology of Ti alloy surface changed by chemical treatment. Leaching of titanium alloy in NaOH results in the formation of a hydrated titanium oxide gel layer containing alkali ions in its surface. This interlayer has thickness up to 5  $\mu$ m. EDS analyses detected small amount of Na<sup>+</sup> ions in the surface (table 3). The amorphous character of TiO<sub>2</sub> layer was confirmed by WAXS-GI XRD method.

Table 2. The ion concentrations of SBF compare to inorganic part of blood plasma (mmol/dm<sup>3</sup>).

	Na <sup>+</sup>	K <sup>+</sup>	Ca <sup>2+</sup>	Mg <sup>2+</sup>	Cl <sup>-</sup>	HCO <sub>3</sub> <sup>-</sup>	HPO <sub>4</sub> <sup>2-</sup>	SO <sub>4</sub> <sup>2-</sup>
plasma	142.0	3.6-5.5	2.1-2.6	1.0	95.0-107.0	27.0	0.7-1.5	1.0
SBF	142.0	5.0	2.5	1.0	131.0	5.0	1.0	1.0

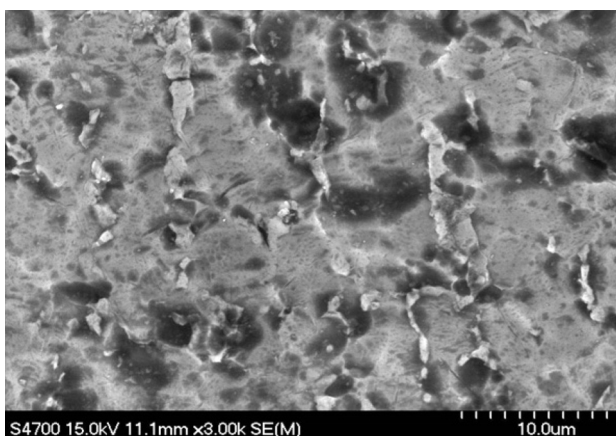


Figure 1. SEM micrograph of Ti alloy original surface.

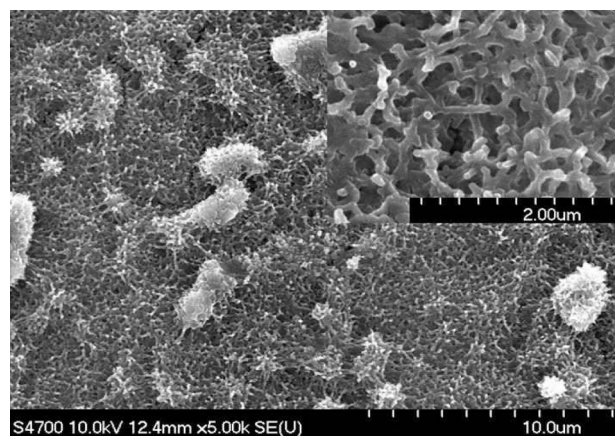


Figure 2. SEM micrograph of Ti surface after treatment in HF, NaOH (detail of the structure in the corner).

Table 3. Chemical composition of the surface before and after chemical treatment.

Method of analyses	Surface treatment	Ti (at.%)	Al (at.%)	V (at.%)	Ti/Al	Ti/V	Al/V	Na (at.%)
SEM-EDS	before	83.5	12.1	4.4	6.9	19.0	2.8	-
SEM-EDS	HF, NaOH	30.0	1.7	0.9	18.2	34.9	1.9	5.5

Analysis of the SCS1-3 solutions after precalcification process

Results of SCS1-3 solutions were published and discussed in [7]. The significant decrease of the  $\text{Ca}^{2+}$  and  $(\text{PO}_4)^{3-}$  ions was detected already after the first day of the exposure in SCS1-2 solutions. The ions consumption stopped after the 4th day of the exposition. We can assume that the nucleation period of the Ca-P precipitation started in the first minutes of the samples exposition and Ca-P phase formed continuously till the solution is supersaturated to precipitated calcium-phosphate. The solution named SCS3 was unstable and calcium-phosphates precipitated before exposition of the Ti-alloy sample.

In vitro test (exposition in SBF solution)

In vitro test compares results of the precalcified samples with the non-precalcified sample in SBF solution. Analysis confirmed that precalcified samples (figures 3b-d) had absorbed  $\text{Ca}^{2+}$  and  $(\text{PO}_4)^{3-}$  ions immediately after immersion till the 7<sup>th</sup> day of exposition in SBF. In the case of the non-precalcified sample (figure 3a) this phenomenon had occurred on the 6<sup>th</sup> day after immersion and finished till the 14<sup>th</sup> day. The residual concentration of the both ions was about 40-50  $\text{mg}/\text{dm}^3$  and subsequently the Ca-P precipitation stopped.

Analysis of SBF solutions after exposure of Ti alloy samples had only informative character (figures 3a-d). In spite of this fact, there is evident phenomenon - shortening of incubation period of forming Ca-P if the samples were precalcified.

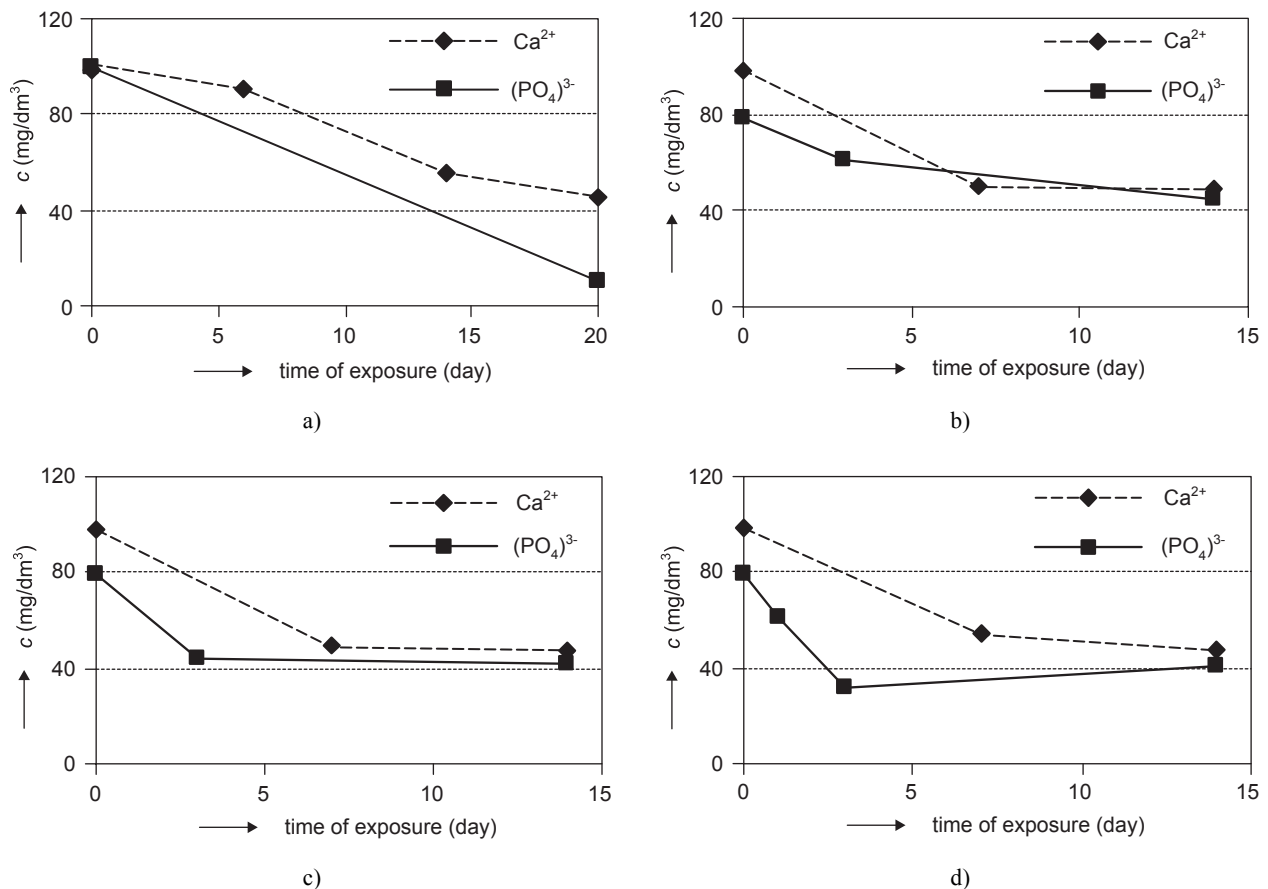


Figure 3. Time dependence of  $\text{Ca}^{2+}$  and  $(\text{PO}_4)^{3-}$  concentrations a) of the non-precalcified sample in SBF (20 days) and precalcified ones in b) SCS1 (7 days), c) SCS2 (7 days), d) SCS3 (7 days) solutions with subsequent exposure in SBF (14 days).

### Study of Ca-P layer on the surface after exposure in SBF

The quality and thickness of the formed layers seems different (figures 4-5). Ca-P spherulites formed on non-precalfified surface are distributed non-homogenously on the sample surface containing of Ca-P layer (figure 4). This fact was confirmed by SEM-EDS [8]. However, only 7 days of exposition in SBF in the case of precalfified sample (SCS2) is enough time for complete covering of the sample surface by homogenous Ca-P layer (figure 5). This layer shows the crystalline character. Needle-like crystals are spherically seated.

Thickness of the Ca-P layer was approximately 5  $\mu\text{m}$  after 20 days in the case of non-precalfified sample and 13  $\mu\text{m}$  on the precalfified surface after 14 days in SBF, respectively (both under static conditions). Studying of the layers by optical microscopy after immersion in SBF confirmed that precalfification treatment had positive influence on the Ca-P growth. The analytical measurement (figures 3a-d) confirmed the layer observations (figures 4-5).

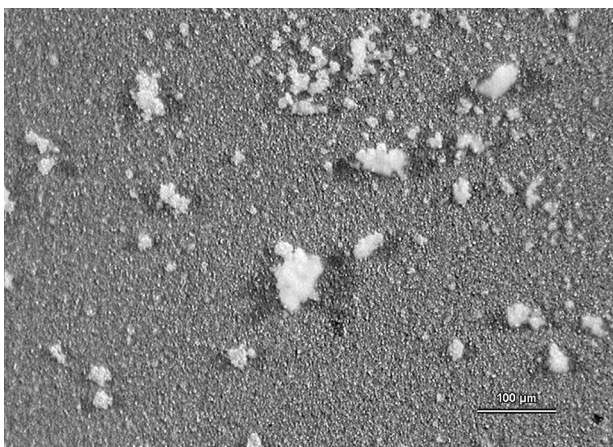


Figure 4. Optical micrograph of the surface of non-precalfified sample after soaking in SBF (20 days).

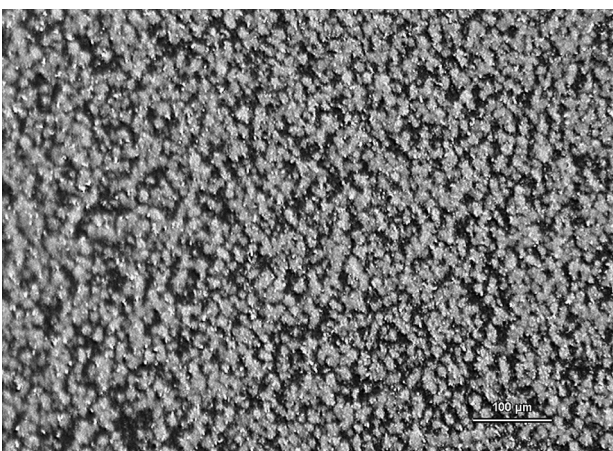


Figure 5. Optical micrograph of the precalfified surface in SCS2 (7 days) with subsequent exposure to SBF (7 days).

It has been concluded that using SCS2 solutions can help to precipitate of the Ca-P phase in SBF solution. Consequently, the Ti alloy surface with nucleated Ca-P phase is good base for next growth of crystalline Ca-P phase in SBF as well as human plasma solutions.

### The effect of $S/V$ ratio on the thickness and quality of Ca-P layer

The SCS2 solution was chosen as the base for the precalfification process with the aim to study the influence of  $S/V$  ratio on nucleation and growth of Ca-P layer. Ti alloy samples were immersed in SCS2 solution under static condition at 37°C for 7 days and at  $S/V = 0.1; 0.3$  and  $0.5 \text{ cm}^{-1}$ .

### Results of precalfification analysis for various aspect of the $S/V$

The results of analysis confirmed (figures 6a-c), that the ions consumption with time of exposure have analogous trend for each  $S/V$  ratio in SCS2 solution. The absorption of ions was stopped on the range of about 160-170  $\text{mg}/\text{dm}^3$  for  $\text{Ca}^{2+}$  and 140-150  $\text{mg}/\text{dm}^3$  for  $(\text{PO}_4)^{3-}$  after the 4<sup>th</sup> day of exposure in SCS2 solution independent on  $S/V$  ratio. Measurement of  $pH$  value confirmed that  $pH$  decreased continuously from 6.4 to 5.8 in all cases. It means that SCS2 solution had become under saturated to Ca-P which is formed on the surface on the 4<sup>th</sup> or 7<sup>th</sup> day, respectively. The optimum time for Ca-P phase formation seems to be the 4<sup>th</sup> day of exposure, when the Ca/P molar ratios (calculated from consumed amount of ions) achieved 1.72; 1.81 and 1.67 (1.67 is the theoretical value for hydroxyapatite) for  $S/V = 0.1; 0.3$  and  $0.5 \text{ cm}^{-1}$ , respectively (figure 6d).

### Study of the Ca-P layers (thickness and morphology) after precalfification process by optical microscopy

Ti alloy surfaces are completely covered by fine grain minerals of Ca-P phase at  $S/V = 0.1 \text{ cm}^{-1}$  as well as  $0.5 \text{ cm}^{-1}$  (figure 7). Differences are in thickness and probably quality of this layer. The thicknesses continuously grow from 25  $\mu\text{m}$  ( $S/V = 0.5 \text{ cm}^{-1}$ ) to 50  $\mu\text{m}$  ( $S/V = 0.1 \text{ cm}^{-1}$ ). Figures 7 and 8 demonstrate the thickness of the layer (cross section) on surface after the 7<sup>th</sup> days of exposure at  $S/V = 0.1$  and  $0.3 \text{ cm}^{-1}$ , respectively. Spherulites formed in SCS2 solution at  $S/V = 0.3 \text{ cm}^{-1}$  do not covered of the sample surface completely. The diameter of these spherulites is approximately of 30  $\mu\text{m}$  (figure 8).

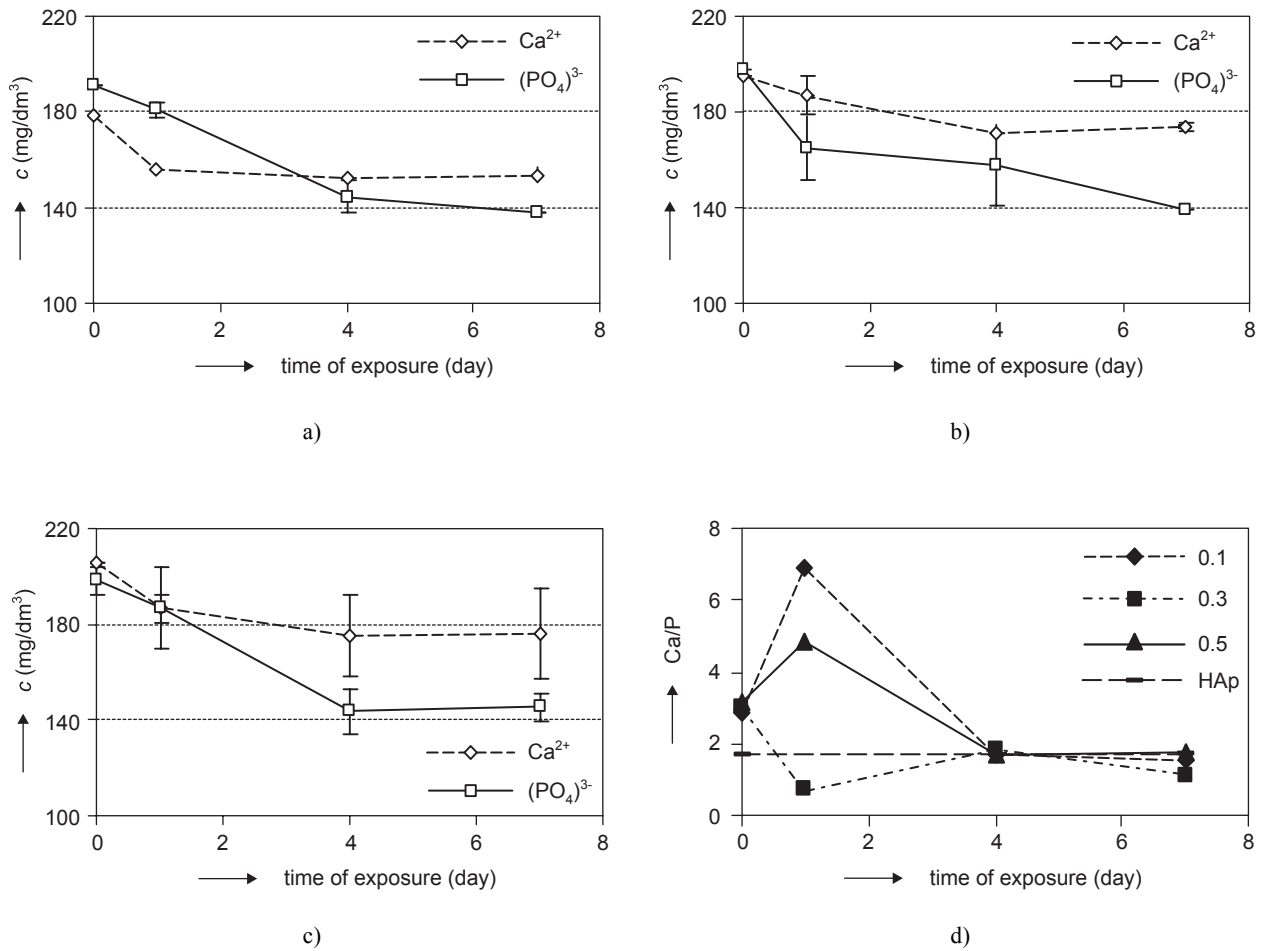


Figure 6. Time dependence of changes in  $\text{Ca}^{2+}$  and  $(\text{PO}_4)^{3-}$  concentration after exposition in SCS2 solution - a)  $S/V = 0.1 \text{ cm}^{-1}$ , b)  $S/V = 0.3 \text{ cm}^{-1}$ , c)  $S/V = 0.5 \text{ cm}^{-1}$ , d) calculated Ca/P molar ratio of consumed ions under various  $S/V$ .

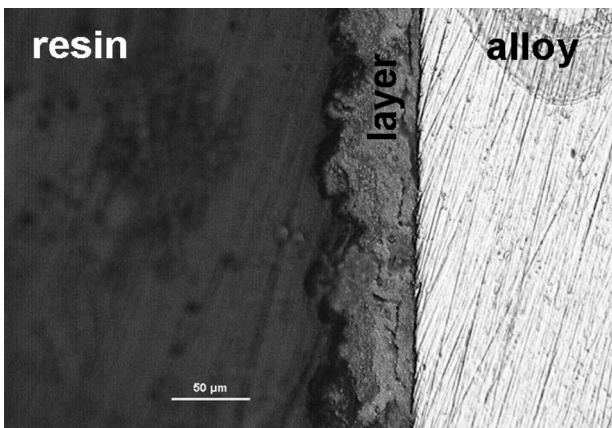


Figure 7. Optical micrograph, cross section of the layer formed in SCS2.

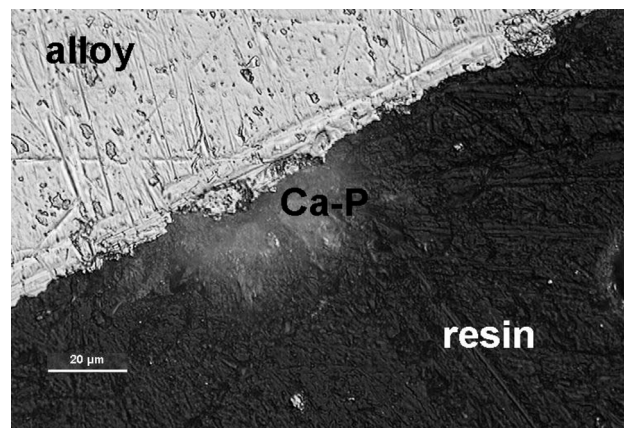


Figure 8. Optical micrograph, cross section of the spherulite.

## CONCLUSION

- 1) Precalcification process has significant influence on shortening of incubation period of Ca-P precipitation in SBF, resulting Ca-P layer is twice thicker compared to the non-precipitated samples.
- 2) The thickness of the Ca-P layer nucleated by precalcification depends on  $S/V$  ratio and can be predicted.
- 3) Precalcification process is finished where the solution became under saturated for formation of Ca-P and in our experiments it was on the 4<sup>th</sup> day, consequently static conditions for in vitro testing are not satisfying because of quick consumption of  $\text{Ca}^{2+}$  and  $(\text{PO}_4)^{3-}$  ions as well as decrease of  $\text{pH}$  value in supersaturated solutions.

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## PŘÍPRAVA KALCIUMFOSFÁTU NA Ti SLITINĚ PREKALCIFIKAČNÍM PROCESEM ZA STATICKÝCH PODMÍNEK

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Bioaktivním materiálem v souvislosti s náhradami kostní tkáně rozumíme materiál, který je schopen za dostatečně krátkou dobu vytvořit s kostní tkání pevnou vazbu. Titanové materiály (Ti6Al4V) využívané k ortopedickým nebo dentálním náhradám jsou materiály inertní. Chemickou úpravou jejich povrchu pomocí silných kyselin a zásad se docílí pokrytí povrchu tenkou vrstvičkou  $\text{TiO}_2$  gelu. Následně několikadenní expozice v přesycených roztocích označených SCS1-3 (vysoký obsah  $\text{Ca}^{2+}$  a  $(\text{PO}_4)^{3-}$  iontů a vhodné  $\text{pH}$ ) se na povrchu gelu nukleuje a sráží dostatečně silná vrstva kalcium-fosfátu. Přítomnost této rozpustné kalcium-fosfátové vrstvičky následně urychlil cca o 6 dnů proces tvorby kalcium-fosfátu při in vitro testech v statickém uspořádání. Prekalcifikace tak může urychlit proces vyhojování. Druhá část práce byla zaměřena na vliv poměru  $S/V$  (plochy vzorku ku objemu kapaliny (SCS2)). Z měření koncentrace iontů  $\text{Ca}^{2+}$  a  $(\text{PO}_4)^{3-}$  vyplynulo, že největší změny se v prekalcifikačním roztoku odehrávají 1. den po vložení vzorku a prakticky ukončují do 4. dne od začátku expozice. Roztok přestává být přesycen vůči kalciumfosfátu, který se na povrchu vysrážel odčerpáním  $\text{Ca}^{2+}$  i  $(\text{PO}_4)^{3-}$  iontů a snížením jeho  $\text{pH}$ . Tloušťka i velikost vzniklých jehlicovitých krystalků sféroliticky uspořádaných je závislá na poměru  $S/V$ . Odčerpané množství Ca i P a jejich poměr se již po 4. dnu blíží teoretickému poměru v HAp. Z naměřených dat také vyplývá, že k testování bioaktivních materiálů je vhodnější tzv. dynamické uspořádání testu, kde bude zaručen přívod čerstvého roztoku SBF (simulated body fluid) po celou dobu testování materiálu.