THE RESORPTION OF ß-TCP AND HA MATERIALS UNDER CONDITIONS SIMILAR TO THOSE IN LIVING ORGANISMS

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The submitted work examined kinetics of formation of a newly formed phase of hydroxyapatite (HAp) on two synthetically prepared bone regenerating materials: β-tricalcium phosphate (TCP) and porous hydroxyapatite (HA). In vitro tests with simulated body fluid (SBF) were performed under a continual flow of fresh SBF solution in partly and fully filled testing cells. It has been found out that in the case of testing cells partly filled (¼ of the volume) with TCP and porous HA the contact between the material and SBF was better and thus also the precipitation of the new phase HAp was faster. The BET method identified a ten times increase of the (originally very small) TCP surface in both cases of cell filling, which indicates precipitation of the HAp phase. For porous HA the newly formed phase (HAp) cannot have been identified with SEM/EDS or RTG diffraction as its character was the same as that of the tested material. However, the BET analysis demonstrated a decrease in the size of the specific area of the porous HA after its exposure in the SBF solutions in both test arrangements, which indicates covering of the material surface with a newly formed HAp phase. Precipitation of the new phase was also confirmed by the increased weight of tested porous HA. The tested materials were not completely covered with the new HAp phase after 13 days of testing and their surface thus remained accessible for further resorption.

INTRODUCTION

Hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$) is, in terms of its chemical composition, similar to the inorganic part of bone tissue. It has been used for regeneration of bone tissue in dental and maxillofacial surgery for defects resulting from bone resorption, extraction or removal of tumors, in treatment of periodontal defects, filling of bone defects in orthopedics, but also in neurosurgery, e.g. as a material filling the vertebral bodies or for replacement of intervertebral discs. Further, it has been applied in form of coatings on inert materials (Ti and its alloys) for the purposes of implants, where higher compression strength is required from the material (hip joint replacements, dental implants) [1-5]. The resorbable materials induce formation of new bone tissue by their dissolving, which results in supersaturation of the blood plasma in respect to hydroxyapatite that starts precipitating in that particular location [1,4]. The biodegradation is also affected by the size of particles and porosity of the material. The ability of interaction of a synthetically prepared HA therefore also depends on the method of its preparation - its sintering phase. It has been found out that kinetics of the formation of the bone apatite decreases with the growing sintering temperature of the synthetically prepared HA [6].

Apart from HA, other materials based on calcium phosphates have been tested for bone replacements. One of the most important is β-tricalcium phosphate (β-TCP, $Ca_3(PO_4)_2$) [7-9].

The initial tests for any material with anticipated clinical use are *in vitro* tests. *In vitro* tests put the materials in contact either with a simulated body fluid (SBF), simulating the inorganic part of blood plasma, or with other testing solutions (e.g. Ringer´s solution) [2, 6,7,9-11]. The resulting reactions include dissolution, precipitation and ion exchange accompanied by absorption and incorporation of biological molecules [7]. Two models have been proposed to explain dissolution of calcium phosphates: the first model is dissolution, where the phenomenon controlling the process is the transport of mass – diffusion model (the driving force is a concentration gradient in the Nernst diffusion equation), and the second model, in which the phenomenon controlling the process is the surface reaction (the driving force is the gradient of potentials

between the surfaces of apatite crystals and the solution). Kinetics of the dissolution depends on many parameters, particularly on the pH value, temperature, exposure time and saturation of the surrounding solution with calcium and phosphate ions [4,5,12]. In the first model the rate of dissolution is therefore controlled by transport of chemical reagents, while the other model uses the rate of the chemical reaction on the apatite surface as the limiting factor [5].

The knowledge of behavior of hydroxyapatite and β-tricalcium phosphate in contact with simulated body fluid is useful for methods of their application as bone tissue replacements. An important question is e.g. the granulometry and the best way of cavity filling with the resorbable material so that it transforms into the new bone tissue as much as possible. The question is whether the entire volume of the cavity should be filled in or only a part of it. It should be also considered which size of particles is the most suitable to ensure good reaction of the filing material with blood plasma so that the resorbable material is completely transformed into the new HAp phase.

The objective of the work was to monitor kinetics of HAp formation on surfaces of synthetically prepared resorbable materials. Porous hydroxyapatite (HA) and porous β-tricalcium phosphate (TCP) were exposed to SBF solution under dynamic conditions at two levels of filling of testing cells. In the first case one quarter of the cell volume was filled and in the second case the cell was filled in completely.

EXPERIMENTAL

The tested materials were prepared by Lasak spol. s.r.o. The first material - resorbable β-tricalcium phosphate (β-TCP, $Ca_3(PO_4)_2$), called Poresorb®-TCP was supplied in form of white granules (1-2 mm) with the specific weight 2 900-3 100 $kg.m^{-3}$ and it contained macro (100-200 μm) and micro (1-5 μm) pores. In the work it is identified as TCP. The second material was hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$), called OSSABASE®-HA and it was supplied in form of white granules (1-2 mm) with submicro pores and macro pores. In the work it is identified as HA. For verification of their bioactivity a model simulated body fluid (SBF) was used, which simulates the inorganic part of human blood plasma with the chemical composition shown in Table 1. The solution was prepared from the following reagents: KCl, NaCl, $NaHCO_3$, $MgSO_4$, $CaCl_2$, K_2HPO_4, the value pH = 7.45 and was buffered with a solution of (Tris-hydroxymethyl aminomethan) and HCl [13].

Table 1. Composition of the simulated body fluid (SBF) [13].

Solution	Ionic concentration (mmol l^{-1})							
	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	HPO_4^{2-}	HCO_3^-	SO_4^{2-}
SBF	142.0	5.0	2.5	1.0	131	1.0	5.0	1.0

The first group of testing cells had one quarter of their volumes filled, for which 1g of TCP material was sufficient. In this work the cells are identified as ¼V TCP. The second group of testing cells contained 4g TCP each - identified as 1V TCP. Similarly, one group of testing cells had one quarter of their volumes filled with the 0.5g of the HA material and they were identified as ¼V HA and the second group of testing cells was filled completely with 1.83g HA each and identified as 1V HA. The volume of each testing cell was 5.5 ml. Throughout the test duration (13 days) a peristaltic pump was used to ensure continual flow of fresh SBF (50 ml per day). The temperature of SBF was maintained with a thermostat at 36.5 ± 0.5°C. In selected time intervals small portions of leachate were collected and analyzed for concentrations of calcium and $(PO_4)^{3-}$ ions and pH value. Experiments were conducted always in two parallel cells for both types of filling and materials.

Surfaces of the samples before and after the exposure were examined with the electron microscope HITACHI S-4700 with EDS analyzer with the accelerating voltage 15kV. RTG diffraction analysis was measured on the diffractometer PANalytical X´Pert PRO at the accelerating voltage 40kV. The specific surface of the granules before and after the exposure was determined with the BET method on the ASAP 2020 device made by Micromeritic, using Kr and N_2 gases. The concentration of calcium ions in the leachates was analyzed with atomic absorption spectrometry using VARIAN-Spectr AA 300. Atomization of samples was performed by means of acetylene-N_2O flame. The absorbance of Ca was measured at the wavelength of 422.7 nm. The content of phosphates was analyzed on the UV-VIS Spectrophotometer UV1601 at λ = 830 nm, in conformity with ČSN 830540. The pH value was measured with the inoLab pH-meter, with a combined glass electrode at the laboratory temperature.

RESULTS AND DISCUSSION

Material Poresorb®-TCP: porous β-Tricalcium Phosphate (TCP)

Measurements of weights of the material before and after the experiment (Tab. 2) have shown that the weight of TCP material increased for two types of cell filling (both ¼V TCP and 1V TCP).

The increase of weight of the TCP material was higher for partial filling of the cell with the sample (¼V TCP), up to two times (4%), in comparison with the completely filled cells (1V TCP) where the weight increased by 2%. This effect can be explained as follows: The smaller quantity of the material was washed with

SBF more evenly and thus better conditions were ensured for the development of a new (probably HAp) phase on the surface of TCP granules.

Table 2. Average* weights of the TCP material before and after its exposure to SBF.

Partly filled testing cell ¼V TCP			
Weight (g)		Increase	
Before	After	Δ (g)	Δ (%)
1.00	1.04	0.04	4
Completely filled testing cell 1V TCP			
Weight (g)		Increase	
Before	After	Δ (g)	Δ (%)
4.00	4.08	0.08	2

** The average value of weights of the materials for two parallel experiments*

The values of specific surface of the TCP material before and after the exposure were measured with the BET method and they are shown in Table 3.

Table 3. Specific surfaces of the TCP material before and after the exposure v solution SBF.

TCP	Specific surface (m^2g^{-1})
Original	0.15*
¼V TCP	1.79
1V TCP	1.35

** measured in Kr*

The results confirm a substantial increase of the surface of the TCP material after the exposure to the SBF solution for both types of filling. The specific surface in the partially filled cell (¼V TCP) increased in comparison with the original value up to twelve times. In the completely filled testing cell (1V TCP) the specific surface increased by the factor of nine.

The following images from the electron microscope show the surface of the TCP material before (Figure 1) and after (Figures 2, 3) its exposure to the SBF solution.

The images (Figures 2, 3) indicate that the surface of the TCP material was after 13 days of exposure to the SBF solution covered with small crystals of calcium phosphate - probably HAp. In the case of the partial filling of the testing cell (¼V TCP) we suppose a better contact of the material with the SBF solution, which has been confirmed by formation of plate-shaped nano-crystals of HAp. Nano-crystals are arranged into spherulites with the size of 5-10 μm (Figure 2). Spherulites are formed preferentially on convex locations, which are energetically more favorable for crystallization of the new phase. In the second case, when the testing cells were completely filled with the material (1V TCP), we suppose that the contact of the material with the SBF solution was imperfect. An image from SEM (Figure 3) demonstrates that only a small quantity of the new phase has developed and the crystals of calcium phosphate are distributed on the TCP surface very unevenly. The plate-shaped nano-crystals are again grouped into spherulites,

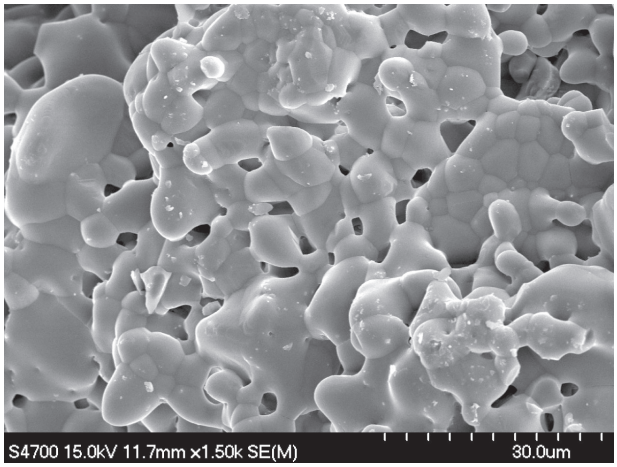

Figure 1. Surface of the original TCP material before the exposure.

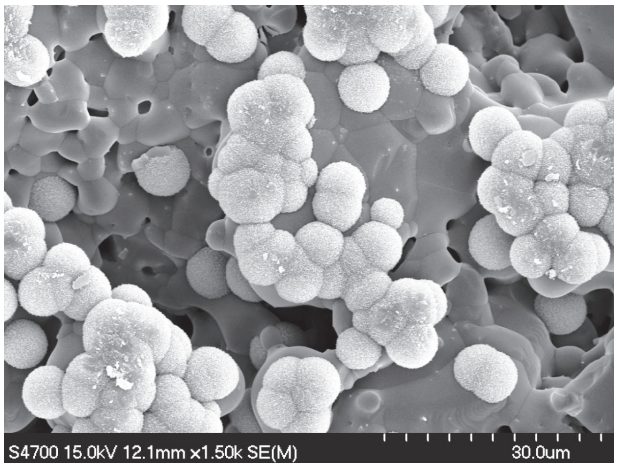

Figure 2. Surface of the material ¼V TCP (partly filled testing cell) after the exposure to SBF with the newly formed HAp phase.

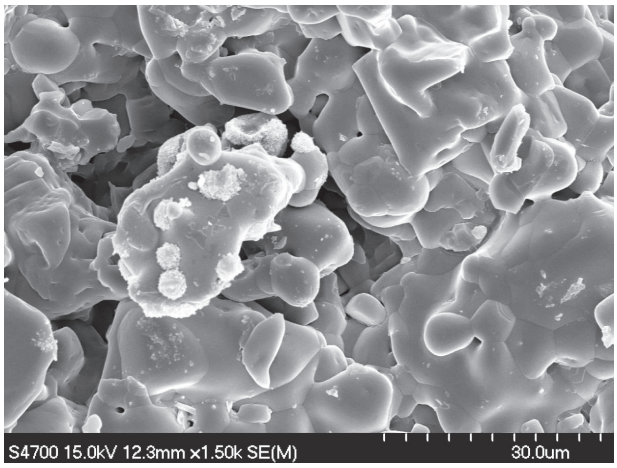

Figure 3. Surface of the 1V TCP material (completely filled testing cell) after the exposure to SBF.

which are much smaller (up to 5µm) than those formed in the case of partially filled testing cells. Also in this case the spherulites formed preferentially on convex locations.

EDS analyses of the surface of the TCP material after its exposure to SBF (¼V TCP and 1V TCP) have shown that the ratio Ca/P is not significantly different from that before the exposure.

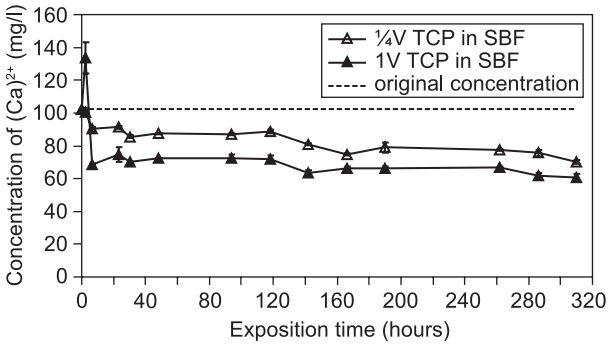

Figure 4. Concentration of calcium ions in the SBF solution for partial (¼V TCP) and complete (1V TCP) filling of the testing cells.

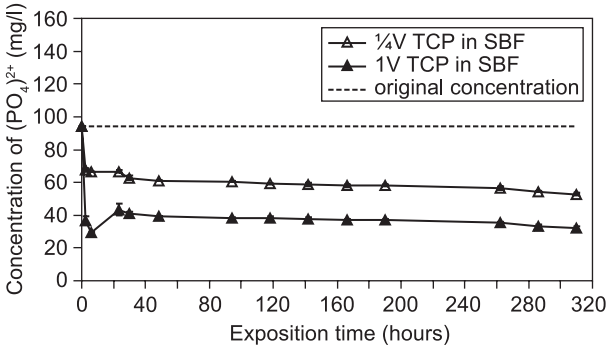

Figure 5. Concentrations of phosphate ions in the SBF solution for partial (¼V TCP) and complete (1V TCP) filling of the testing cells.

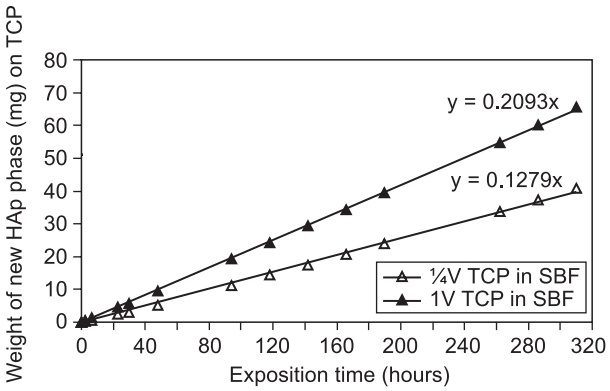

Figure 6. Weight increases of TCP calculated from decreases of concentration of $(PO_4)^{3-}$ ions for partial (¼V TCP) and complete (1V TCP) filling of the testing cells.

RTG difractograms of the TCP material (Ref. Code: 01-070-2065) after its exposure to the SBF solution (¼V TCP and 1V TCP) were not different from those of the original material. This has been probably due to the small quantity of the newly formed phase in comparison with the original material.

Figures 4 and 5 show the time dependence of Ca^{2+} and $(PO_4)^{3-}$ concentrations in SBF in the course of the material exposure for both types of filling (¼V TCP and 1V TCP). Figure 6 shows weight increases of TCP (i.e. the weight of the new HAp phase) in the course of the exposure, calculated from decreases of concentration of $(PO_4)^{3-}$ ions in the leachates.

Table 4 contains precipitation rates of the HAp phase on the TCP surface which were calculated from the actual weight increases and decreases of $(PO_4)^{3-}$ ions from the beginning of the exposure (equations (1)-(3)). A substantial decrease of the $(PO_4)^{3-}$ ions in the leachate from the beginning of the exposure (up to 2 hours) actually indicates immediate precipitation of the new HAp phase.

From the weight of the new phase:

$$R_{\mathrm{HAp}} = (m_{\mathbf{TCP}(\mathrm{final})} - m_{\mathbf{TCP}(\mathrm{initial})}) / t \qquad (1)$$

From the decrease of concentration of $(PO_4)^{3-}$ ions:

$$c_{(\mathrm{PO4})}{}^{3-} = k_{\mathrm{HAp}}\ t \qquad (2)$$

Relative rate of the precipitation:

$$R'_{\mathrm{HAp}} = R_{\mathrm{HAp}} / m_{\mathbf{TCP}(\mathrm{initial})} \qquad (3)$$

where R_{HAp} is the rate of formation of the new HAp phase, calculated from the increased weight; $m_{\mathbf{TCP}(\mathrm{final,\ initial})}$ are weights of TCP after and before the exposure, respectively; $c_{(\mathrm{PO4})}{}^{3-}$ is the concentration of phosphate ions in SBF leachates; k_{HAp} is a constant determined from the line slope (Fig. 6); R'_{HAp} is the rate of precipitation of the new HAp phase related to a unit of weight; t is the time of exposure.

Table 4. Rate of precipitation of the HAp phase on the surface for ¼V TCP and 1V TCP in SBF.

Filling of the cell with the TCP material	R_{HAp} (mg hour^{-1})	k_{HAp} (mg hour^{-1})	R'_{HAp} (mg hour^{-1}g^{-1})
¼V TCP - 1 g	0.129	0.128	0.129
1V TCP - 4 g	0.258	0.209	0.065

Analyses of SBF solutions (Figures 4 and 5) have suggested that the TCP material dissolves significantly from the very beginning of the test: the concentration of Ca^{2+} ions in the solution increases within 2 hours while the concentration of $(PO_4)^{3-}$ ions decreases. Those contrary trends suggest that after its exposure TCP dissolves immediately and this causes a substantial supersaturation of the SBF solution in respect to HAp and, at the same time, the new HAp phase precipitates from the SBF solution. Within a short period of time (within 24 hours

for both types of cell filling) a stable removal of Ca^{2+} and $(PO_4)^{3-}$ ions from the SBF solution occurred, i.e. a stable precipitation of HAp. The diagram indicates that in the partly filled cell (¼V TCP) the removal of Ca^{2+} and $(PO_4)^{3-}$ ions is less intense than in the completely filled cell (1V TCP). This phenomenon corresponds well with the overall quantity of the tested material inside the cell. The weight of the sample in a partly filled cell is one quarter of that in a completely filled cell (Tab. 2) and therefore the increase of a newly forming HAp phase is higher in the case of the partly filled cell (¼V TCP) than in the completely filled cell (1V TCP) (Tab. 4). The values of pH of the SBF solution for both types of filling did not differ significantly.

The diagram showing dependence of weight increases of the TCP material (Fig. 6) indicate that the weight increase during the 13 days of exposure to SBF is linear. The rate of formation of the new HAp phase is calculated from the beginning of the exposure. The relative rate of HAp precipitation in the partly filled cell (¼V TCP) is twice higher when compared to the completely filled cell (1V TCP). The results indicate the new apatite layer on the TCP material is formed more readily in the cell which was only partly filled with the material. However, a question remains whether the fast formation of HAp on the TCP surface is a desirable process from the viewpoint of its resorbability. If the surface is covered too fast the further resorption of the material may significantly slow down or stop completely.

Material OSSABASE®-HA: porous hydroxyapatite (HA)

The dynamic conditions, under which an interaction between porous hydroxyapatite and SBF was monitored, were the same as with the previous material. The weight of materials was determined before and after the experiment for two parallel measurements and it was found out that also in case of porous hydroxyapatite (HA) its weight after the experiments increased (Tab. 5). Similarly as with TCP**,** we observed a twice higher increase of the new phase in respect to the initial weight for the partially filled cell (¼V HA).

Table 5. Weight of HA before and after the exposure to SBF*.

Partly filled testing cell ¼V HA			
Weight (g)		Increase	
Before	After	Δ (g)	Δ (%)
0.5	0.6	0.1	20
Completely filled testing cell 1V HA			
Weight (g)		Increase	
Before	After	Δ (g)	Δ (%)
1.83	2.0	0.17	9.2

** The average value of weights of the materials for two parallel experiments*

Equally as with the previous material (TCP), this significant difference can be explained for the porous hydroxyapatite (HA) by a better contact of the SBF solution with the smaller quantity of the material.

The values of the specific surface of the porous hydroxyapatite before and after the exposure in the SBF solution were measured with the BET method and they are shown in Table 6.

Table 6. Specific surfaces of the porous HA before and after the exposure in the SBF solution.

HA	Specific surface ($m^2\ g^{-1}$)
Original	70.79
¼V TCP	61.34
1V TCP	67.05

The results indicate a decrease in the values of the specific surface after the exposure to the SBF solution for both test arrangements. A higher decrease (up to 13.4 %) was found for the material in a partly filled cell (¼V HA), which suggests formation of a bigger quantity of the new nano-crystalline HAp phase.

The following images from the electron microscope show the HA surface before (Figure 7) and after (Figures 8, 9) its exposure to the SBF solution.

The comparison of images from SEM have shown that the surface of the porous hydroxyapatite partly changed after its exposure. Figure 8 shows a strongly broken relief of the hydroxyapatite in the case of the partly filled cell (¼V HA). Rod-shaped crystals were found on the surface which formed bigger agglomerates (up to 4 μm). In the completely filled cell (1V HA) the quantity of agglomerates was lower (Figure 9).

The results from the EDS analysis did not show a significant difference in the composition of the hydroxyapatite surface before and after the exposure to the SBF solution.

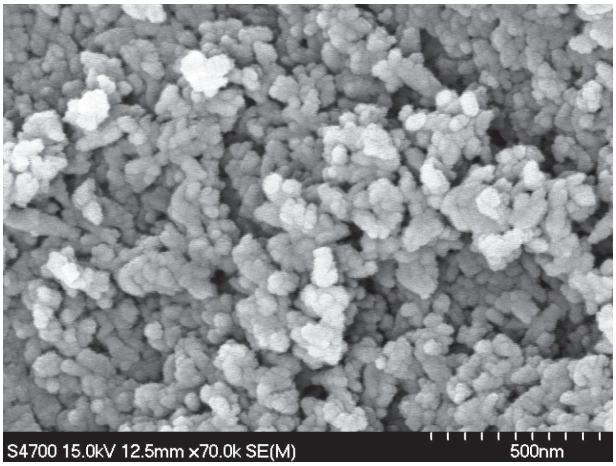

Figure 7. Surface of the original hydroxyapatite (HA) material before the exposure.

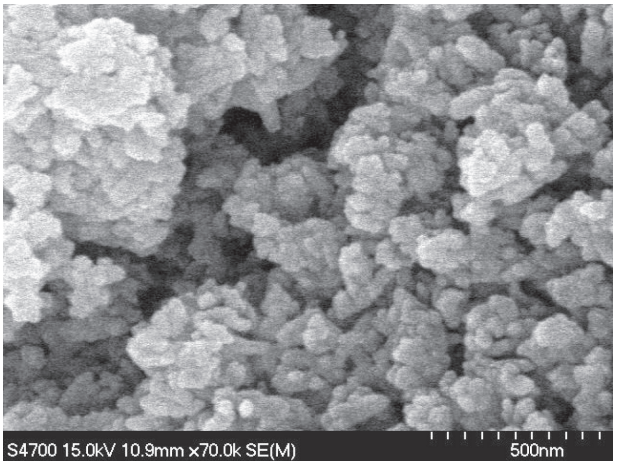

Figure 8. Surface of the ¼V HA material (from the partly filled testing cell) following the exposure to SBF.

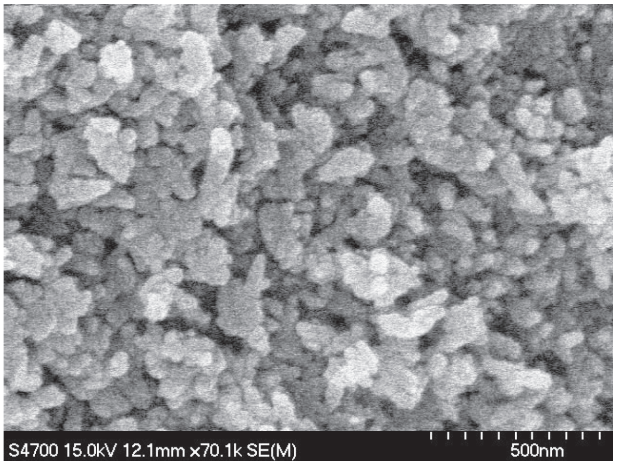

Figure 9. Surface of the material 1V HA (completely filled testing cell) after the exposure to SBF.

Again, the diffractogram ¼V HA was not different from the original hydroxyapatite (Ref. Code: 01-089-6439), which is logical considering the composition of the expected new phase - the hydroxyapatite. In the completely filled cell 1V HA a small difference was found in the stoichiometry of the newly formed HAp (Ref. Code: 01-089-6438) in comparison with the tested porous HA, which suggests formation of a defective HAp.

Figures 10 and 11 show of Ca^{2+} and $(PO_4)^{3-}$ concentrations in SBF during the exposure.

Figure 12 shows the linear dependence of the growth of weight of the tested porous hydroxyapatite in both test arrangements (¼V HA and 1V HA), calculated from the decrease of $(PO_4)^{3-}$ ions. Table 7 contains precipitation rates of the new phase (HAp) on the porous HA surface, as calculated from the slope of the lines for ¼V HA and 1V HA.

Figure 10 indicates that concentrations of calcium ions in SBF are similar at the beginning of the exposure for individual types of test arrangements, as it was in the case of TCP. For the partly filled cell (¼V HA) the concentration of Ca^{2+} decreased immediately from the very beginning which indicates that the precipitation of the new HAp phase from the SBF solution is faster in comparison with the dissolution of the porous phase HA. In the case of the completely filled cell (1V HA) the concentration of calcium ions increased at the beginning (within 24 hours) which suggests a massive dissolution of the HA material.

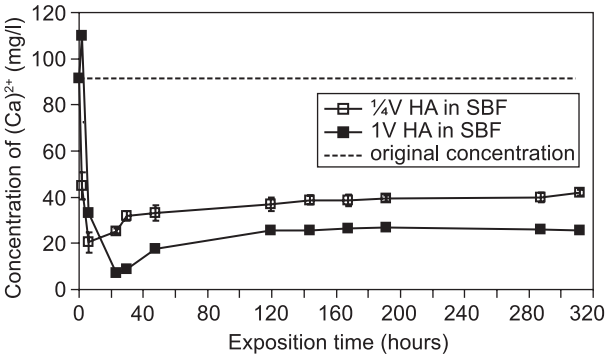

Figure 10. Concentrations of calcium ions in the SBF solution in the partially (¼V HA) and completely (1V HA) filled testing cells.

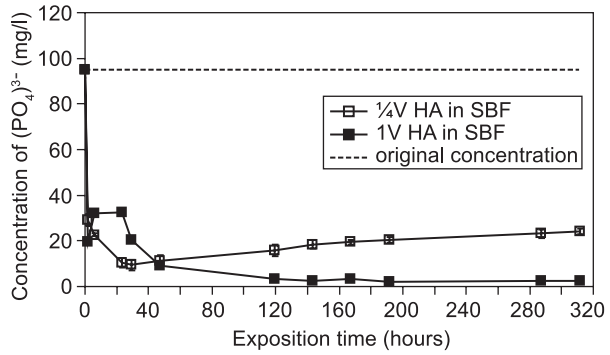

Figure 11. Concentrations of phosphate ions in the SBF solution in the partially (¼V HA) and completely (1V HA) filled testing cells.

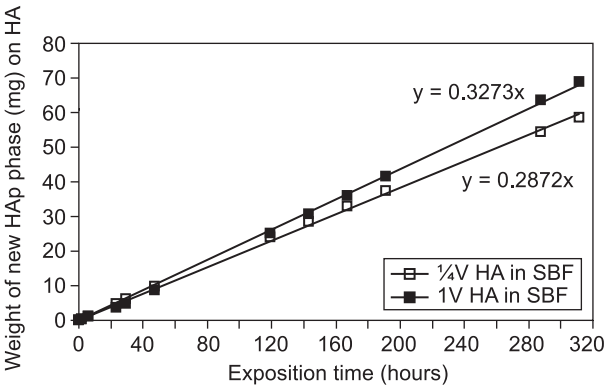

Figure 12. Weight increases of HA (i.e. new HAp phase) for partial (¼V HA) and complete (1V HA) filling of the testing cell.

In the case of $(PO_4)^{3-}$ ions (Figure 11) their concentrations at the beginning of the exposure decreased for both test arrangements. The decrease was more significant in the fully filled cell (1V HA) where the concentration of $(PO_4)^{3-}$ ions decreased substantially (from 95 mg l^{-1} to 19 mg l^{-1}). Subsequently, the removal of ions stabilized and practically all $(PO_4)^{3-}$ ions contained in SBF were continually removed. After approximately 60 hours the rates of ion removal and the precipitation of the new phase became stable in both test arrangements.

Relative precipitation rates (mg.hour^{-1}.g^{-1}) shown in Table 7 indicate a high reactivity of the HA surface with the simulated body fluid. A higher quantity of the precipitated HAp phase in [mg.hod^{-1}] in the case of the HA material has been achieved thanks to the enormously large specific surface of HA in comparison with TCP. The surface of HA per one gram is up to 470 times larger than that of TCP.

Table 4. Precipitation rates of the HAp phase on the surface for ¼V HA and 1V HA in SBF.

Filling of the cell with the HA material	R_{HAp} (mg hour^{-1})	k_{HAp} (mg hour^{-1})	R'_{HAp} (mg hour^{-1})
¼V HA - 0.5 g	0.320	0.287	0.640
1V HA - 1.83 g	0.545	0.328	0.174

** The symbols are explained in the section on TCP (Table 4)*

CONCLUSION

1. For both the HA and TCP materials it was found out that the precipitation of the new HAp phase is more intense in case of partial filling of the cell with the material (¼V**).**
2. The precipitation rate of new HAp phase allows to do a conclusion that the precipitation proceeds continually for both types of materials.
3. Analyses of Ca^{2+} and $(PO_4)^{3-}$ ions in SBF leachates for both materials clearly show that the processes of dissolution and precipitation of the new phase became stable after 24 hours of the exposure for TCP and after 60 hours of exposure for HA.
4. The tests have shown that the materials were not completely covered with the new (nearly insoluble) HAp phase after 13 days of the experiments and therefore the resorption process may continue.
5. Further experiments will seek to identify conditions for the maximum resorption of the materials, by extending the experiment duration or by changing the set-up conditions of the dynamic test.

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