THE INFLUENCE OF SIMULATED BODY FLUID COMPOSITION ON CARBONATED HYDROXYAPATITE FORMATION

ALEŠ HELEBRANT, LENKA JONÁŠOVÁ, LUDVÍK ŠANDA

Department of Glass and Ceramics,
Institute of Chemical Technology, Prague,
Technická 5, 166 28 Prague, Czech Republic
E-mail: Ales.Helebrant@vscht.cz

Submitted June 6, 2001; accepted October 1, 2001.

The materials for bone and dental implants are tested in vitro using simulated body fluid (SBF). The composition of most used SBF differs from that of blood plasma by high content of Cl^- ions and lower content of HCO_3^- ions. Considering the composition of bone-like apatite, which contains carbonate ions, the test results could be influenced by this difference. The glass of system Na_2O-CaO-SiO_2-P_2O_5 was leached in fluids of composition (in mmol/l): 142 Na^+, 5 K^+, 2.5 Ca^{2+}, 1 Mg^{2+}, 1 SO_4^{2-}, 1 HPO_4^{2-}, 136 (Cl^-+HCO_3^-). The content of HCO_3^- ranged between 5 and 27 mmol/l. The decrease of phosphorus concentration was observed after 2 days in all solutions indicating the creation of phosphate layer on the glass surface. X-ray diffraction (XRD) confirmed the various extent of apatite layer creation in different solutions. The intensity of XRD peaks seems to be more influenced by the initial solution supersaturation than by the supersaturation values during the interaction. This fact indicates that the nucleation could be the control mechanism of apatite precipitation. Both experimental and supersaturation results indicate that the SBF with increased amount of HCO_3^- ions is more appropriate and sensible to in vitro testing of bioactive materials.

Keywords: Simulated body fluids, In vitro testing, Bioactive materials, Hydroxyapatite

INTRODUCTION

The artificial materials considered for implants are tested by *in vivo* methods (in live animal organism) and by *in vitro* ones (in media simulating the body fluid). The tests are focused on their physical, chemical and mechanical properties and thus provide the basic information allowing the suitability of a material for implanting into the human organism to be assessed.

In the in vitro tests, the material is exposed to the effects of aqueous solutions simulating the inorganic part of blood plasma in the presence or absence of cell cultures, and the interactions of the surface with the solution are examined. Both ground and compact samples are tested, and changes in concentration of individual components in the model solutions, and also changes in the surface of the samples are studied.

As the conditions of the actual experiments described in the literature have not yet been standardised, it is always necessary to specify precisely the temperature, composition and *pH* of the leaching solution, the time and way of the exposure (the conditions are mostly static), the ratio of the material surface area to the volume of the leaching solution (*S/V*), etc. The solution designated SBF (Simulated Body Fluid) in the literature is among the most favoured model solutions simulating just the inorganic part of the human blood plasma. A comparison of the concentrations of ions (mmol/l) in human plasma and in SBF is given in table 1 [1–3]. In the literature, mention is further made of Ringer´s solution, of the "HBSS" model solution and of the „199 medium" model solution (table1). The "HBSS" contains 1 g/l of D-glucose and the "199-medium" 0.4 g $Ca_3(C_6H_5O_7)4H_2O$, 0.885 g alkaline phosphatase, 0.03 g thymol blue a 0.05 g phenol red per litre of solution [4].

The composition of the most used SBF differs from that of human blood plasma by high content of Cl^- and lower content of HCO_3^- ions. Considering the composition of bone-like apatite, which contains

Table 1. Comparison of the concentration of ions in blood plasma and in other solutions (mmol/l).

	Na^+	K^+	Mg^{2+}	Ca^{2+}	Cl^-	HCO_3^-	HPO_4^{2-}	SO_4^{2-}
blood plasma	142.0	3.6-5.5	1.0	2.1-2.6	95.0-107.0	27.0	0.65-1.45	1.0
SBF	142.0	6.5	1.5	2.5	148.0	4.2	1.0	0
Ringer´s sol.	39.1	1.4	0	0.4	40.7	0.6	0	0
HBSS	141.7	5.7	0.8	1.7	145.6	4.2	0.7	0.8
199-medium	0	0	0	27.8	0	9.8	27.1	0

carbonate ions, the test results could be influenced by this difference.

The aim of the present study was to prepare the modified simulated body fluids with different concentrations of the above-mentioned ions and to discuss the influence of these simulated fluids composition on the apatite formation.

EXPERIMENTAL

The samples employed

The test were carried out with glass of this composition (wt.%): 24.5 Na_2O, 24.5 CaO, 45 SiO_2, 6 P_2O_5. The experiments were performed on compact samples with dimensions $1 \times 1.5 \times 0.2$ cm. Their surface was treated by grinding in ethanol-based solution with diamond disks using 50, 20 and 10 μm grain size.

Exposure of the glass to model solutions

The composition of used model solutions is given in table 2.

Table 2. Composition of the model solutions (mmol/l).

	SBF5	SBF10	SBF15	SBF20	SBF27
Na^+	142.0	142.0	142.0	142.0	142.0
K^+	5.0	5.0	5.0	5.0	5.0
Ca^{2+}	2.5	2.5	2.5	2.5	2.5
Mg^{2+}	1.0	1.0	1.0	1.0	1.0
Cl^-	131.0	126.0	121.0	116.0	109.0
HCO_3^-	5.0	10.0	15.0	20.0	27.0
SO_4^{2-}	1.0	1.0	1.0	1.0	1.0
HPO_4^{2-}	1.0	1.0	1.0	1.0	1.0

The fluids were prepared by dissolving of KCl, NaCl, $NaHCO_3$, $MgSO_4 \cdot 7H_2O$, $CaCl_2$ and KH_2PO_4 in distilled water and were buffered with TRIS (50 mmol/l). *pH* was adjusted with HCl to 7.2–7.3 at 37°C, i.e. the value close to the *pH* of the human blood plasma (7.23). NaN_3 was added into the each solution (1 g/l) to inhibit the bacteria formation.

The samples were exposed to the solutions under static conditions at 37°C ± 0.4°C for at the most 14 days. The samples were freely suspended in a volume of model solution roughly corresponding to $S/V \cong 0.05$ cm^{-1}. After exposure, the samples were washed off by distilled water.

Analyses of the extracts and evaluation of glass surfaces

Interactions of glasses with eluting solutions were evaluated on the basis of analyses of the extracts and by determining the changes in the glass surface (X-ray diffraction analysis). To evaluate the ability and rate of hydroxyapatite formation on the glass surface, changes in the concentration of phosphates and calcium were determined. It may be assumed that decreasing amounts of these components indicate the formation of a layer enriched with P and Ca on the glass surface. However, it is necessary to take into account that the changes in concentration of $(PO_4)^{3-}$ and in particular of Ca^{2+} are at the same time affected by transfer of the components from glass into solution, insofar as the respective components are present in the glass.

The concentrations of $(PO_4)^{3-}$ in extracts were determined spectro-photometrically, and the concentrations of Ca^{2+} by the AAS method.

The composition of glass surfaces was analysed by the Seifert 3000P X-ray diffractometer, using CoKa radiation and graphite monochromator.

RESULTS AND DISCUSSION

Stability of model solutions

In the first experiments, the stability of the model soaking solutions was evaluated by the time dependence of $(PO_4)^{3-}$ and Ca^{2+} ions concentration. It may be assumed that concentration decrease of these components indicates the spontaneous precipitation and the instability as well. The results are shown in figure 1.

The changes in $(PO_4)^{3-}$ and Ca^{2+} solution concentration were negligible and did not exceed the analytical error (±3.9 mg/l). The *pH* values changes were maximal in SBF27 (figure 1*c*). Nevertheless, the change was smaller than 0.2. The results obtained show that all of the solutions are stable and no spontaneous precipitation occurs.

Glass-solution interaction

The time dependence of $(PO_4)^{3-}$ and Ca^{2+} concentration in various soaking solutions is shown in figure 2. The increase of $(PO_4)^{3-}$ was noted only after 1 day of exposure to SBF20, i.e. transfer of $(PO_4)^{3-}$ from glass into solution prevailed over precipitation. After longer time of exposure only the decrease of $(PO_4)^{3-}$ was determined in all solutions. The highest $(PO_4)^{3-}$ concentration decrease was detected in SBF5 and SBF10 after 14 days of soaking. In other solutions (SBF15, SBF20, SBF27) the decrease of $(PO_4)^{3-}$ concentration was smaller (about 30 mg/l). Presented results indicate that only after 2 days of soaking a formation of P-rich layer occurs.

The time dependence of Ca^{2+} concentration shows that no decrease in calcium concentration in solutions occurred even after 14 days, i.e. extraction prevailed over precipitation. The highest increase of Ca^{2+} was noted in the SBF27 (about 40 mg Ca^{2+}/l), the lowest one in the SBF5 (about 20 mg Ca^{2+}/l). The results indicate that it is not possible to confirm the Ca-rich surface layer formation from the Ca^{2+} solution concentration.

X-ray diffraction spectra of P45 glass after the interaction with different model solutions are shown in figure 3. Hydroxyapatite was identified on the surface

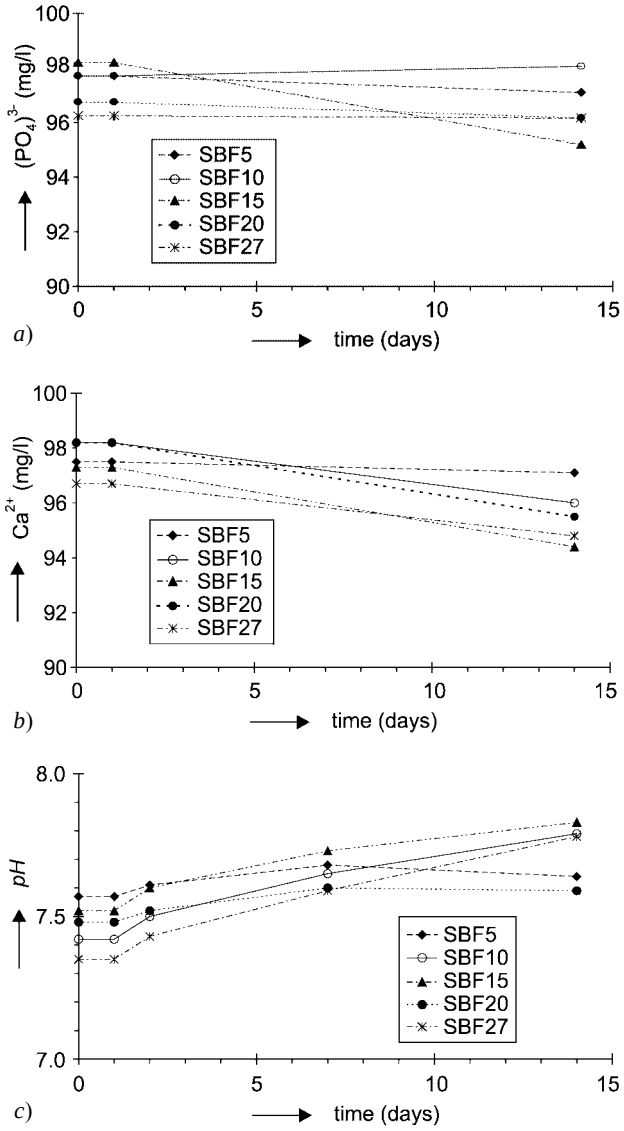

Figure 1. The stability of model solutions.
a) time dependence of $(PO_4)^{3-}$ concentration, *b*) time dependence of Ca^{2+} concentration, *c*) time dependence of solution *pH* (25°C)

of each sample after 14 days of interaction with model solutions. The slight shift of characteristic peaks was observed comparing the results with reference spectra of carbonated hydroxyapatite (HCA, figure 3*b*) and hydroxyapatite (HA, figure 3*c*). This result suggests the differences in the structure of precipitated layer in different solutions. The diffusive character of observed peaks is result of poor crystallinity of the precipitated product.

Compared to others, the XRD spectrum of P45 sample soaked in SBF27 was the most diffuse one. This fact indicates low crystallinity of the precipitate caused most probably by relatively short time of exposure in SBF and/or smaller thickness of precipitated layer.

No proportionality was found between the XRD peaks intensity and the decrease of phosphate concentration in the solution. This fact could indicate differences in precipitation mechanism and in composition of precipitated layer in different model solutions. Due to the composition most similar to the blood plasma, the solution SBF27 should be used for the in vitro testing. The slower creation of precipitated apatite layer in comparison with other solutions could enable the more sensitive in vitro testing of bioactive materials. Moreover, during the interaction with human blood plasma the creation of carbonated hydroxyapatite could be awaited rather than pure hydroxyapatite precipitation. Therefore, the content of carbonate ions in the solutions can be important for the plausibility of in vitro test.

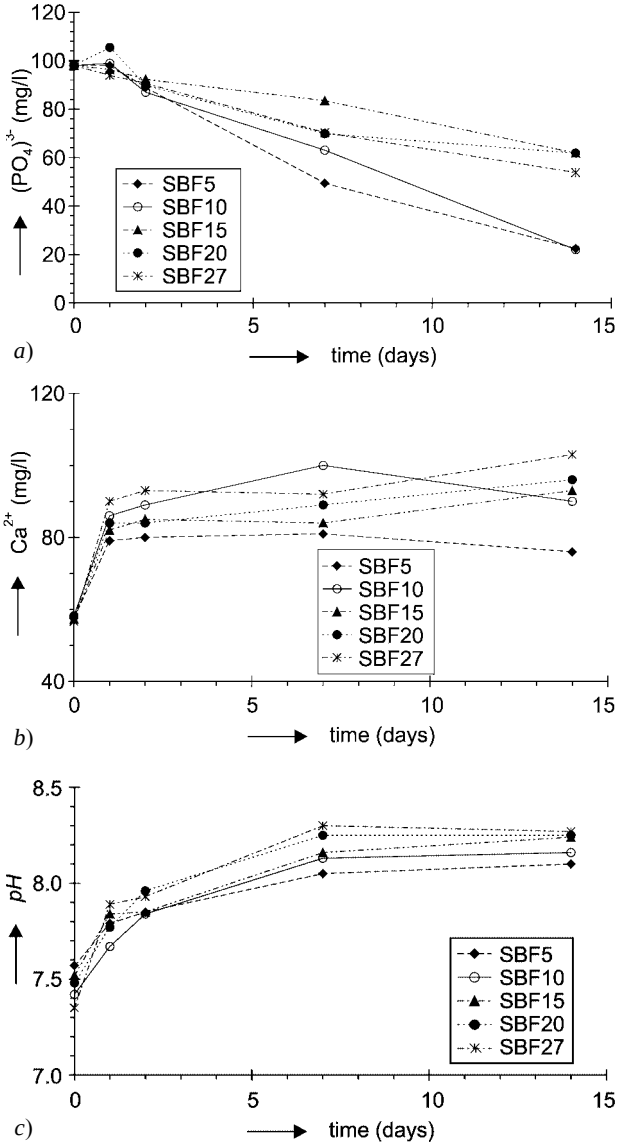

Figure 2.The solution changes during interaction with bioactive glass P45.
a) Time dependence of $(PO_4)^{3-}$ concentration, *b*) Time dependence of Ca^{2+} concentration, *c*) Time dependence of solution *pH* (25°C)

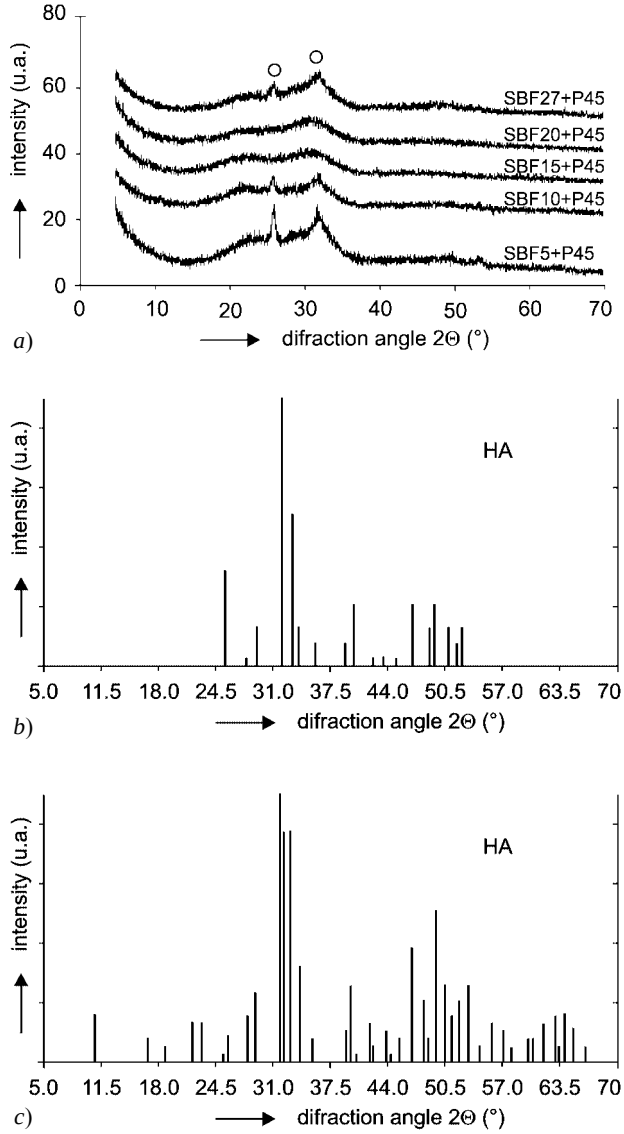

Figure 3. X-ray-diffraction patterns.
a) layer precipitated on the P45 glass surface after 14 days of interaction with simulated body fluids. The compositions of SBF used are summarised in table 2. The circles mark the hydroxyapatite peaks, *b*) reference sample of carbonated hydroxyapatite (HCA), *c*) reference sample of hydroxyapatite (HA)

Solution supersaturation

The relative supersaturation s with respect to different types of phosphates was computed using the equation

$$\sigma = \sqrt[n]{\frac{IAP}{K}} - 1 \qquad (1)$$

where *IAP* means ion activity product, *K* solubility product, *n* number of ions in precipitate formula. The supersaturation values are depicted in figure 4. The formulae of compounds discussed and the values of solubility products are summarised in table 3.

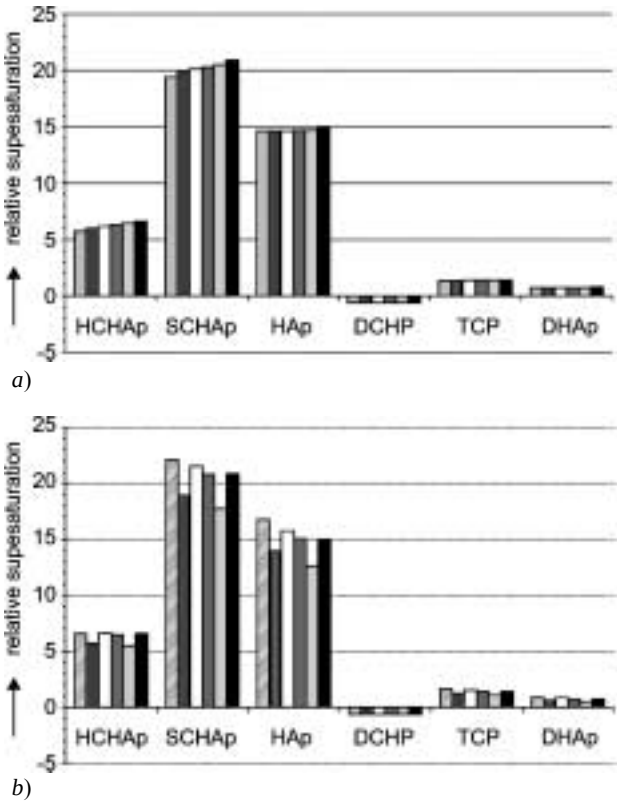

Figure 4. The supersaturation of solutions studied with respect to different phosphates. *a*) assuming the same *pH* value (7.25) for all solutions, *b*) considering *pH* values measured at $t = 0$

Table 3. The solubility products of different phosphates.

formula	name	-log *K*	Ref.
$Ca_{10}(PO_4)_6CO_3$	heavily carbonated apatite HCHAp	102.8	[5]
$Ca_{10}(PO_4)_6(CO_3)_{0.5}OH$	slightly carbonated apatite SCHAp	115.6	[5]
$Ca_{10}(PO_4)_6(OH)_2$	hydroxyapatite HAp	117.2	[6]
$CaHPO_4.2H_2O$	calcium hydrogenphosphate dihydrate DCHP	6.0	[7]
$Ca_3(PO_4)_2$	tricalcium phosphate TCP	28.6	[7]
$Ca_9(HPO_4)(PO_4)_5OH$	defective hydroxyapatite DHAp	85.1	[6]

With exception of DCHP, the solutions are supersaturated with respect to all phosphates discussed. Assuming the theoretical value of *pH*, the differences in supersaturation are negligible excepting HCHAp and SCHAp according to the content of carbonate ions in the solution. The computed supersaturation does not respond to the XRD results. However, considering the *pH* values measured at the beginning of interaction in each solution, the supersaturation degree is changed (figure 4*b*) and is considerably lower for SBF10 and SBF27. This result is in agreement with XRD data. On the other hand, the time dependence of relative supersaturation with respect to HAp (figure 5) suggests that the rate of precipitation should be higher for SBF15, SBF20 and SBF27 than for SBF5 and SBF10. This is in contrary to experimental results. It seems that the rate of apatite layer creation is significantly influenced by the initial supersaturation whereas the effect of supersaturation in further stages of interaction is much smaller. This fact could be explained by controlling role of nucleation. It is in agreement with our previous study [8] where the polynucleation was determined as the controlling mechanism of apatite precipitation on glass and glassceramics.

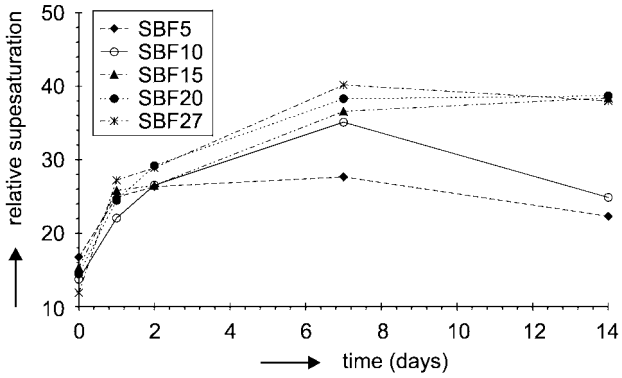

Figure 5. The time dependence of relative supersaturation with respect to hydroxyapatite during the interaction of glass P45 with different solution.

CONCLUSIONS

The simulated body fluids with different contents of chloride and carbonate ions were tested. The stability of all solutions enables in vitro testing of bioactive materials without spontaneous homogeneous precipitation.

The hydroxyapatite was detected on the surface of bioactive glass P45 after 14 days of interaction with all solutions tested. The XRD patterns and the changes in phosphate content in the solutions suggest the different thickness and crystallinity of the precipitated layer. These differences can be explained by the different supersaturation of bulk solutions at the beginning of interaction. This fact indicates the important role of nucleation in the apatite layer creation. The precipitation and crystallisation is smaller in the model solution SBF27 with carbonate content near to the human blood plasma compared to the SBF5, which is often used to the in vitro testing of bioactive materials. Therefore, SBF27 could be more suitable and sensitive for the in vitro testing of bioactivity.

Acknowledgement

This study was supported by the Grant Agency of the Czech Republic under the project number GACR 106/97/0957. The authors also thank to Dr.Had for providing the XRD measurements. The hospitality of Trinity College and invaluable discussions with Dr.Jan Czernuszka from the Department of Materials during Aleš Helebrant's stay at The University of Oxford in summer 1997 helped us in this study.

References

1. Fujiu K., Ogino M., Kariy T., Ichimura T.: J. Non-Crystal. Solids *56*, 417 (1983).
2. Terao N.: Silicate industrieles *52*, 123 (1987).
3. Homolka J.: *Clinical biochemistry in use* (in Czech), 1982.
4. Weinstain A.M., Klawitter J.J., Cook S.D.: J. Biomed. Mat. Res. *14*, 23 (1980).
5. I to A., Maekawa K., Tsutsumi S., Ikazaki F., Tateishi T.: J. Biomed. Mater. Res. *36*, 522 (1997).
6. Driessens F.C.M.: *Mineral Aspects of Dentistry*, p.14, S.Karger, Basel 1982.
7. Nancollas G.H. in: *Biomineralization*, p.159, Editors Mann S., Webb J., Williams R.J.P., VCh Verlagsgesellschaft mbH, Weinheim 1989.
8. Helebrant A., Rohanová D.: Glastech. Ber. Glass Sci. Technol. *67C*, 111 (1994).

VLIV SLOŽENÍ MODELOVÝCH ROZTOKŮ NA TVORBU POVRCHOVÉ VRSTVY KARBONÁTOVÉHO HYDROXYAPATITU

ALEŠ HELEBRANT, LENKA JONÁŠOVÁ, LUDVÍK ŠANDA

Ústav skla a keramiky
Vysoká škola chemicko-technologická v Praze
Technická 5, 166 28 Praha

Umělé materiály uvažované pro implantace jsou testovány zkouškami in vivo (v živém organismu zvířete) a in vitro. Při testech in vitro je testovaný materiál vystaven působení vodných roztoků, které simulují anorganickou část krevní plazmy s obsahem nebo bez přítomnosti buněčných kultur a sledují se interakce povrchu materiálu s roztokem. Mezi nejvíce používané modelové roztoky patří roztok označovaný v literatuře zkratkou SBF (Simulated body fluid), který simuluje pouze anorganickou část lidské krevní plazmy. Dále je v literatuře uváděn Ringerův roztok, modelový roztok "199 medium" a roztok označovaný "HBSS" (Hanks´ balanced

salts solution). Uvedené modelové roztoky se liší v koncentraci některých iontů (Cl^-, HCO_3^-) od složení anorganické části lidské krevní plazmy.

Cílem předkládané práce bylo připravit takové modelové roztoky, aby se svým složením co nejvíce přiblížily složení anorganické části lidské krevní plazmy a dále ověřit, zda jsou tyto roztoky schopné simulovat tvorbu hydroxyapatitové vrstvy na povrchu bioaktivních materiálů.

U skel soustavy Na_2O-CaO-SiO_2-P_2O_5, která byla loužena v pěti různých modelových roztocích, byl sledován vliv složení těchto roztoků na tvorbu povrchové vrstvy HA. Simulované tekutiny byly připraveny tak, že s rostoucím obsahem HCO_3^- klesal obsah Cl^-. Koncentrace Na^+, K^+, Mg^{2+}, Ca^{2+}, $(HPO_4)^{2-}$ a $(SO_4)^{2-}$ byly ve výchozích roztocích shodné. Všechny roztoky byly během expozice v biologickém termostatu stálé, nedocházelo k jejich srážení. Ve výluzích byly sledovány změny pH, koncentrace $(PO_4)^{3-}$ a Ca^{2+}. K úbytku fosforu z roztoku docházelo při nízkém poměru *S*/*V* již po 2 dnech expozice. Z této změny je možné usoudit na jeho zpětné srážení v povrchu testovaných skel a tak na vznik vrstvy obohacené o fosfor. Povrchy vzorků po expozici v modelových roztocích byly hodnoceny RTG difrakční analýzou, která potvrdila tvorbu povrchové vrstvy HA.

Výsledky získané analýzou výluhů a RTG difrakcí vedou k závěru, že navrhované modelové roztoky jsou schopné simulovat tvorbu HA na povrchu bioaktivních materiálů.