STUDY OF BIOACTIVE GLASSES BY "IN VITRO" TESTS

PART 1 – ASSESSMENT OF GLASSES ON THE BASIS OF ANALYSES OF EXTRACTS INTO MODEL SOLUTIONS

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Changes in PO_4^{3-} content in extracts from glass into simulated body fluid following various times of extraction were studied on glasses of the system Na_2O-CaO-SiO_2 both with and without a phosphorus content. Following the exposure, the glass surfaces of the samples were evaluated by X-ray diffraction analysis and by IR spectroscopy. The results showed that the changes in phosphorus concentration in the simulated solution allowed glasses of various composition to be assessed with respect to formation of hydroxyapatite layers on the surface.

INTRODUCTION

Damaged organs or parts of the human body are capable of self-reproduction, but sometimes they have to be replaced by implants. These are made of artificial materials of organic or inorganic nature. Prior to their clinical application they have to undergo a number of special tests. On the long-term basis, emphasis is laid on their biocompatibility, that is physical, chemical and physiological stability. Moreover, the materials must not bring about any harmful reactions in live tissues and have to retain the required mechanical properties.

The artificial materials considered for implants are tested by in vivo methods (in live animal organisms) 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 terms of reactivity with live tissues, the implant materials can be divided into inactive ones (whose behaviour approaches that of inert substances) and surface-active materials. The latter, which form direct bonds with live tissues, are called bioactive materials.

Bioactivity was first described [1] in connection with glasses of specific composition in the system Na_2O-CaO-SiO_2-P_2O_5 and subsequently also with that of phosphorus-free glasses and other materials. Formation of hydroxycarbonate apatite, which mediates the bond with live tissue on the surface of the implant, is regarded as the common characteristic of the bioactive materials. The rate of hydroxyapatite formation on the implant surface in contact with the body fluid is a particularly important factor of the bioactivity of a material. The so-called relative bioactivity serving for comparing the degree of bioactivity was devised and described in the literature [1]. It is determined by in vivo tests on the basis of time required for creating a bond with the bone over more than 50 % of the implant surface area.

In the in vivo tests the material is exposed to the effects of aqueous solutions which simulate 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 standardized, 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^{-1}) in human plasma and in SBF is given in table 1 [2, 3]. In the literature mention is further made of Ringer's solution (2.25 g NaCl, 0.105 g KCl, 0.045 g $CaCl_2$ and 0.05 g $NaHCO_3$ per litre of solution), and also of the "199 medium" model solution (3.109 g $CaHPO_4.2H_2O$, 1.328 g $CaCO_3.2H_2O$, 0.885 g H_3PO_4, 0.4g $Ca_3(C_6H_5O_7).4H_2O$, 0.885 g alkaline phosphatase, 0.885 g thymol blue and 0.05 g phenol red per litre of solution).

During the course of the exposure tests the change in *pH* of the solution may influence the rate of the solid-liquid interaction. One of the possibilities of eliminating this effect is the use of a suitable buffering agent. TRIS (tris-hydroxymethyl aminomethane) in combination with HCl is frequently used for this purpose.

According to the authors of [5] the results of in vivo and in vitro tests are not completely comparable. The main cause of the deviations in the behaviour of materials under the conditions of the two types of tests is considered to be due to the absence of all the corresponding organic components in the in vitro tests. The organic substances may affect reactivity of the material and formation of the surface layer.

Table 1. Comparison of the concentration of ions in blood plasma and in SBF (mmol l^{-1}).

	Na^+	K^+	Mg^{2+}	Ca^{2+}	Cl^-	HCO_3^-	HPO_4^{2-}
Blood plasma	142.0	5.0	1.5	2.5	103.0	27.0	1.0
SBF	142.0	6.5	1.5	2.5	148.0	4.2	1.0

Table 2. Composition of the glasses.

Glasses	Composition of glasses (wt.%)			
	SiO_2	Na_2O	CaO	P_2O_5
S45	45	27	27	0
P45*	45	24.5	24.5	6
S50	50	25	25	0
P50	50	22	22	6
S55	55	22.5	22.5	0
P55	55	19.5	19.5	6
P60	60	17	17	6

* the composition of the glass corresponds to glass 45S5 after Hench [1].

The present study had the aim to assess the viability of in vitro tests for evaluation of bioactive materials. In the literature, bioactivity is defined on the basis of in vivo tests as the ability of materials to combine with live tissue as a result of formation of a hydroxyapatite surface layer. From the standpoint of in vitro testing, relative assessment of bioactive materials should therefore be based on comparing their ability to form hydroxyapatite on their surface and the rate of its forming, by following the changes in concentration of the decisive components (Ca, P) in solution after interaction of the material with the model medium, and by evaluating the changes in the surface of the material and those in the surface layers. The available literature does not include any study dealing with the practical usefulness of this type of tests, nor with the evaluation of the methods employed. The present study was therefore focused on evaluating the results of in vitro tests aimed at their utilization as a tool of relative assessment of bioactive materials, using their ability to form hydroxyapatite layers as the criterion.

EXPERIMENTAL PART

The samples employed

The tests were carried out with glasses of the system Na_2O-CaO-SiO_2 with or without a constant addition of P_2O_5 and with a variable content of SiO_2. The glasses and their chemical compositions are listed in table 2. The experiments were performed on compact as well as on ground samples. The surface of the compact samples (dimensions 1 × 1.5 × 2 cm) was treated by grinding in ethanol-based solution with diamond disks using 50 μm and 20 μm grain size. The ground glass samples were prepared to the ČSN ISO 719 standard, employing the 0.3 - 0.5 mm grain size fraction in the tests.

Exposure of the glasses to model solutions

The SBF simulated body fluid, whose composition approaches that of the inorganic part of the human blood plasma, was used as the basic model solution (cf. table 1). The solution was buffered with TRIS and the *pH* was adjusted with HCl to 7.2 - 7.3 at 37 °C, i.e. the value close to the *pH* of the body fluid (7.23). For the purpose of assessing the role of phosphorus, which passes into solution by leaching from the glass, phosphate-free SBF, aqueous SBF solution and distilled water were also tested. Except for H_2O, the *pH* of the solutions was adjusted with HCl to 7.3 - 7.4 at 37 °C.

The samples were exposed to the solutions under static conditions at 37 °C ± 0.4 °C for at the most 12 days. In the case of ground samples, 0.4 g of the glass were placed in polythene bottles and poured over with 44 ml of the respective solution. According to the literature, for the ground samples this corresponds to an approximate ratio of glass surface area to leaching solution volume, *S/V* of 1 cm^{-1}. In the case of compact samples their dimensions were measured and their surface area calculated. The samples were freely suspended in a volume of model solution roughly corresponding to *S/V* = 0.13 cm^{-1}.

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, IR reflection spectroscopy). For the purpose of evaluating 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 are indicative of 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 P and in particular of Ca 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 spectrophotometricaly, and the concentrations of Ca^{2+} by the AAS method.

The composition of glass surfaces was analyzed by the Seifert 3000P X-ray diffractometer, using CoKα radiation and graphite monochromator. IR spectra were measured on the Nicolet 740 spectrometer by the diffusive reflection method.

RESULTS AND DISCUSSION

Comparison of glasses on the basis of analyses of extracts

For the purpose of assessing the interaction of the glasses with the model fluid following short-term exposure, the ground glass samples were exposed to SBF at a high ratio of the glass surface area to the volume of the eluting solution (*S/V* about 1 cm^{-1}). Changes in the concentration of phosphates were already noted within a short period of time, and there were also differences between glasses with and without P_2O_5. With glasses S45 and S55, losses in PO_4^{3-} concentration in solution were already established within 3 or 5 hours of exposure. The glasses were free from phosphorus so that the decrease in the concentration of phosphates in solution was not affected by transfer of phosphorus from glass into solution and may be assumed to be have been due to precipitation of phosphates on the glass surface (figure 1). A comparison of the results for S45 and S55 glasses indicates that with glasses having a lower SiO_2 content (S45) the phosphorus-enriched surface layer forms somewhat faster than on the glass with a higher SiO_2 content (S55).

In the case of glasses containing phosphorus, this passes into solution and at the same time precipitates on the glass surface. The differences between the behaviour of the two glasses is illustrated by a comparison of results obtained for glass couples with identical SiO_2 contents (S45 and P45; S55 and P55), as shown by figure 1. In contrast to phosphorus-free glasses, no greater loss in phosphorus content in solution was noted with phosphate-containing glasses during short-term exposure. The first distinct loss in phosphorus content was noted with P45 glass after 24 hours of leaching. For the short times of exposure, the amount of phosphorus extracted from glass into solution is obviously somewhat balanced by the amount of phosphorus lost by precipitation in the form of hydroxyapatite on the glass surface. With glasses having a higher SiO_2 content (P55, P60) the phosphorus content in solution was at first rising and decreased only after longer exposure; with glass P45 after 12 hours, and with glass P60 after as much as 12 days of leaching. Transfer of phosphorus from glass into solution thus prevails here over its reprecipitation during the early stages of exposure. These results also indicate that with glasses having a higher SiO_2 content the formation of the phosphorus-enriched surface layer is slower than with glasses with a lower silica content.

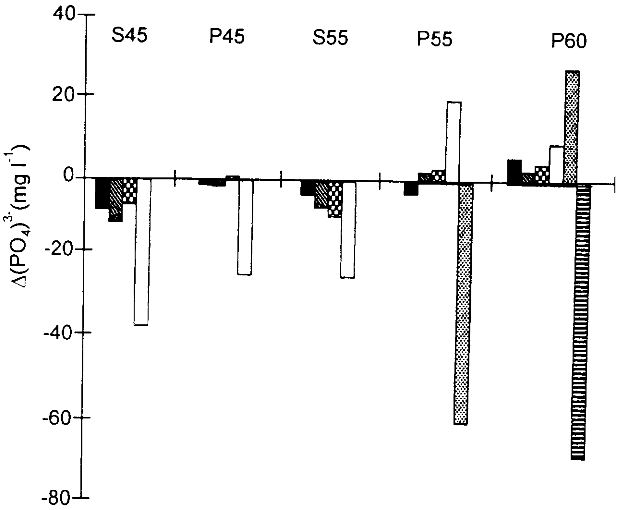

Figure 1. Comparison of changes in PO_4^{3-} concentration in leachates into SBF in terms of time of exposure.
Ground samples; *S/V* approx. 1 cm^{-1}. ■ – 3 hours, ▨ – 5 hours, ▩ – 7 hours, ☐ – 24 hours, ▦ – 5 days, ▤ – 12 days

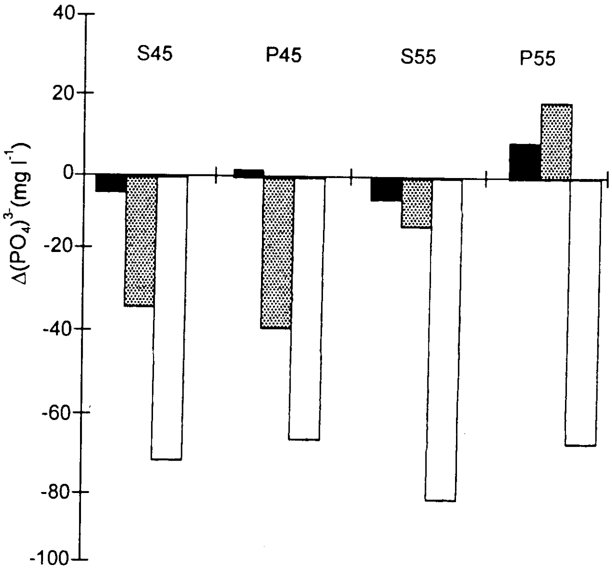

Figure 2. Comparison of changes in PO_4^{3-} concentration in leachates into SBF in terms of time of exposure.
Compact samples; *S/V* approx. 0.13cm^{-1}. ■ – 5 hours, ▦ – 24 hours, ☐ – 5 days

The results obtained show that on the basis of determining changes in the concentration of phosphates in extracts into SBF it is possible to compare glasses in terms of their capability and rate of formation of a phosphate enriched surface layer at a high *S/V* ratio (ground samples) already after short times of exposure.

Similar measurements were also carried out on compact samples but at a lower *S/V* ratio of about 0.13 cm^{-1}. A comparison of changes in PO_4^{3-} concentration in extracts into SBF from compact glass samples is shown in figure 2. The results likewise show that the changes in PO$_4^{3-}$ concentration in leachates from compact samples yield similar conclusions with respect to assessing the formation of a hydroxyapatite layer on individual glasses as do those from ground glass samples.

With both types of glass samples the concentration of Ca^{2+} in solution is also affected by simultaneous extraction of calcium from glass and its reprecipitation. The changes in calcium concentration in leachates into SBF following the exposure of samples were determined with selected glasses only in cases when a significant change in PO$_4^{3+}$ concentration had been established. With all of the glasses studied, during short-term exposure the calcium concentration was increasing in terms of the time of exposure. No loss in Ca^{2+} concentration in the solution occurred after even 24 hours of leaching with any of the glasses involved. In the course of short-term exposure, extraction of calcium therefore prevailed over its reprecipitation on the surface.

The changes in calcium concentrations in the leachates are thus more distinctly affected by extraction of calcium from glass than by the changes in phosphorus content. Measuring the changes in phosphorus content is therefore more suitable for assessing the formation of the hydroxyapatite surface layer, especially in the case of short-term exposure.

Assessment of the effect of phosphorus content in glass

For the purpose of assessing the role of phosphorus passing from the glass into solution during the exposure, ground samples of glasses P45 and P55 were exposed at *S/V* of approx. 1 cm^{-1} to SBF free of phosphates. It was found that with both glasses the initial increase in PO$_4^{3+}$ content in solution is followed by its decrease (figure 3). These results indicate that reprecipitation on the glass surface of phosphorus extracted from the glass takes place under these conditions (figure 3).

Similar measurements were carried out by exposure of glass P45 to an aqueous solution of TRIS alone and the results were similar to those obtained with phosphate-free SBF (figure 4). In contrast to this, exposure of the same duration to distilled water alone resulted in an only slight increase in PO$_4^{3+}$ content in the solution instead of its decrease (figure 4).

The results obtained are in agreement with data in the literature [6] according to which a phosphorus-enriched layer may form on the surface of glasses even in phosphate-free solutions if the bioactive glass contains phosphorus.

Compared to extraction in distilled water alone, the results of exposure to aqueous TRIS solution indicate (figure 4) that the presence of TRIS affects the concentration of PO$_4^{3+}$ in the solutions and thus probably also the formation of a hydroxyapatite layer on the surface of glass. This problem will be the subject of a subsequent publication [7].

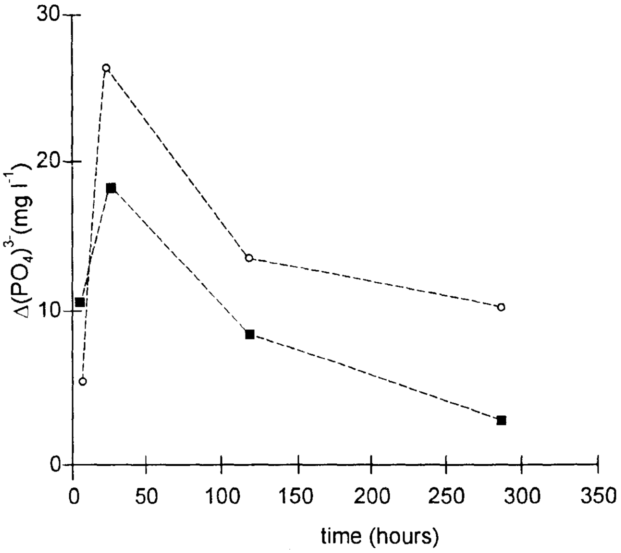

Figure 3. Time dependence of changes in PO$_4^{3-}$ concentration in leachates into phosphate-free SBF.
Glasses P45, P55; ground samples, *S/V* approx. 1 cm^{-1}. ■ – P45, O – P55

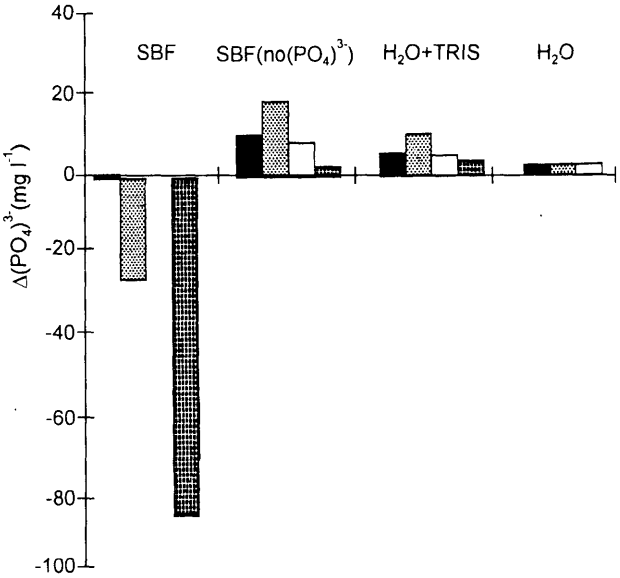

Figure 4. Comparison of changes in PO$_4^{3-}$ concentration in leachates into various model solutions.
Glass P45; ground samples; *S/V* approx. 1 cm^{-1}. ■ – 7 hours, ▦ – 24 hours, ☐ – 5 days, ▩ – 12 days

Evaluation of the surface layers of glasses

The results of analyses of the leachates were supplemented with evaluation of the glass surfaces by X-ray diffraction analysis and by IR spectroscopy. Compact specimens of phosphorus-containing glasses (P50 and P45) and those of phosphorus-free glasses (S50) were used in the experiments.

On the surface of the P50 glass, hydroxyapatite was identified after 5 days of exposure to SBF containing TRIS, and calcite in the case of glass S50. No crystalline phase was found on the glasses within 24 hours of exposure (table 3). Hydroxyapatite was likewise identified on the phosphorus-containing glass (P50) after 5 days of exposure to phosphate-free SBF.

Table 3. The crystalline phases identified in the surface of glasses by X-ray diffraction and IR spectroscopy.

Glass	simulated fluid	time of exposition (hours)	X-ray difraction	IR spectroscopy
P50	SBF	24	0	HA
		120	HA	HA
	SBF no PO_4^{3-}	120	HA	HA
P45	SBF	5	not measured	HA?
		24	not measured	HA
S50	SBF	24	0	HA
		120	calcite	calcite

IR reflex spectroscopy bore out the results of analyses made by X-ray diffraction and allowed the crystalline phases in the sample surface to be identified within shorter times of exposure. Hydroxyapatite was identified on the surface of the P50 sample exposed to SBF + TRIS already after 24 hours (table 3). With phosphorus-containing glasses having a lower SiO_2 content, the first traces of hydroxyapatite lines were already discernible after 5 hours of exposure to SBF + TRIS. In the case of phosphorus-free glasses (S50), hydroxyapatite was also identified after 24 hours of leaching in SBF + TRIS; after longer exposure (5 days) calcite was likewise found, which is in agreement with the results of X-ray analyses (table 3). Following the exhaustion of phosphorus in the solution, calcite will obviously begin to precipitate on the glass surface. This result bears out the conclusions drawn in the previous study [6] in that after longer exposure and at a higher *S/V* ratio, phosphorus-free glasses showed a tendency to calcite formation on the surface of precipitated hydroxyapatite layer.

The results obtained by evaluation of the glass surfaces by X-ray diffraction and IR spectroscopy correspond to the results of analyses of extracts and substantiate the assumption that changes in the phosphorus content can serve as an indication of hydroxyapatite layer formation on the surface of glasses.

CONCLUSION

To be able to evaluate the bioactivity of a material it is important to assess its ability to form a hydroxyapatite layer on its surface when in contact with the body fluid; the rate of this formation process is likewise a significant factor.

The results presented in this study allow to conclude that determination of changes in phosphorus concentration in leachates into simulated body fluids by in vitro testing permits glasses of different compositions to be compared, thus providing first information on the materials with respect to their bioactivity.

Acknowledgement

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References

1. Hench L.L.: J. Am. Ceram. Soc. *74*, 1487 (1991).
2. Fujiu K., Ogino M., Kariy T., Ichimura T.: J. Non-Crystal. Solids *56*, 417 (1983).
3. Terao N.: Silicates industrieles *52*, 123 (1987).
4. Weinstain A.M., Klawitter J.J., Cook S.D.: J. Biomed. Mat. Res. *14*, 23 (1980).
5. Kokubo T., Ito S., Huang Z.T., Hayashi T., Sakka S.: J. Biomed. Mat. Res. *24*, 331 (1990).
6. Rybaříková L., Had J., Holubová I.: Ceramics-Silikáty *39*, 1 (1995).
7. Jonášová L., Rybaříková L.: to be published in Ceramics-Silikáty.

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STUDIUM BIOAKTIVNÍCH SKEL TESTY "IN VITRO"

ČÁST 1 - HODNOCENÍ SKEL NA ZÁKLADĚ ANALÝZ VÝLUHŮ DO MODELOVÝCH ROZTOKŮ

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Na povrchu bioaktivních materiálů se po reakci s tělní tekutinou vytváří vrstva hydroxyapatitu, jejímž prostřednictvím dochází k vazbě s živou tkání. Pro relativní posouzení bioaktivity materiálů pomocí testů in vitro je proto vhodné porovnávat jejich schopnost a rychlost tvorby vrstvy hydroxyapatitu na povrchu sledováním změn koncentrací rozhodujících složek (P, Ca) v roztoku simulujícím tělní tekutinu a hodnocením povrchových vrstev.

U skel soustavy Na_2O-CaO-SiO_2 s obsahem i bez obsahu fosforu (tab.2) byly sledovány změny koncentrací PO_4^{3-} ve výluzích ze skel do simulované tělní tekutiny SBF (tab.1). U skel bez obsahu fosforu byly zaznamenány úbytky PO_4^{3-} v roztoku (obr.1 a 2) již po krátkých časech loužení. U skel s vyšším obsahem SiO_2 byly naopak pro kratší časy expozice naměřeny přírůstky fosforu v roztoku a pokles se projevil až po delších časech loužení (obr.1 a 2). Výsledky analýz výluhů vystihují skutečnost, že u skel obsahujících fosfor dochází současně k přechodu fosforu ze skla do roztoku a k jeho srážení na povrchu skla. U skel s vyšším obsahem SiO_2 dochází zřejmě pomaleji k poklesu obsahu fosforu v roztoku SBF, což indikuje pomalejší tvorbu povrchové vrstvy hydroxyapatitu.

Hodnocení povrchu loužených vzorků RTG difrakční analýzou a IČ spektroskopií potvrdilo výsledky analýz výluhů o tvorbě vrstvy hydroxyapatitu na povrchu loužených skel (tab.3).

Tvorba vrstvy byla prokázána u skel obsahujících fosfor i po expozici do SBF bez fosforečnanů (obr.3, 4 a tab.3) a do vodného roztoku TRISu (obr.4).