

STUDY OF BIOACTIVE GLASSES BY "IN VITRO" TESTS

PART 2 – EFFECT OF TRIS BUFFER ON TEST RESULTS

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The effect of the TRIS buffer on formation of the apatite surface layer was studied on glasses of the system $\text{Na}_2\text{O-CaO-SiO}_2$ both containing phosphorus and free of it. The changes in the specimen surfaces following exposure to model solutions were evaluated by X-ray diffraction analysis and IR spectroscopy. Concentrations of PO_4^{3-} , Ca^{2+} and SiO_2 were determined in the extracts. The results showed that TRIS speeds up significantly the extraction of glasses and simultaneously formation of the hydroxyapatite layer on the glass surface.

INTRODUCTION

Prior to introduction of a biomaterial into the human organism its properties are determined by *in vivo* tests, that is in a live animal organism, and by *in vitro* tests, i.e. in a medium simulating the body fluid.

In the *in vitro* tests, in particular the long-term ones, it is important to keep the *pH* of model solutions unchanged and they are therefore buffered.

It is known from the literature that when working with glass a number of organic compounds generally used in buffering may form complex compounds with metallic elements contained in glass, thus influencing the test results. This fact was pointed out e.g. by Paul [1] who dealt with formation of the complexes for instance in the case of citrates, acetates and EDTA. For examples, citrates and EDTA produce very stable complex compounds with metallic elements (Ca, Pb, and Al) and may thus be assumed to have an accelerating effect on the corrosion of glass. An important role in the process is played by *pH* of the solution.

The solutions modelling the body fluid *in vitro* tests mostly use the TRIS buffer, i.e. tris(hydroxymethyl)aminomethane in combination with HCl. The effect of TRIS on corrosion of glass has not yet been dealt with in the literature, although it is generally used in biochemical laboratories where solutions come into contact with glass. However, study of bioactive glass-ceramic materials revealed a distinct speeding up of transfer of some components (Ca, SiO_2) from these materials into simulated body fluid as well as into distilled water buffered by this agent [2]. The authors explain this fact by formation of soluble complexes of TRIS with calcium ions. At the same time they assume that the presence of TRIS leads to suppressed formation of hydroxyapatite by reducing the oversaturation of the solution and keeping its *pH* at a constant value.

In another study [3] a bioactive glass was tested in aqueous solutions buffered with TRIS combined with HCl or citric acid. Its authors found that on the surface of a specimen exposed to a solution containing TRIS and citric acid, i.e. one with a content of citrate ions, no layer enriched with calcium and phosphorus was formed contrary to the solution buffered with TRIS and HCl in which the layer was produced. The authors explain the phenomenon by formation of soluble complexes of citrate ions with calcium. However, unlike the authors of study [2] the latter authors do not consider any effect of TRIS alone on accelerating the dissolution of glass. At the same time they suppose that citrate has no destructive influence on the silicate lattice of glass. On the contrary, Bacon and Raggon [4] assume that apart from forming complexes with metallic elements, TRIS in a neutral medium also forms complexes with silicon, and that this results in more distinct dissolution of glass than that caused by formation of complexes with the ions of the modifiers. These assumptions are also borne out by the results given in [5].

The present study was aimed at assessing the effect of TRIS as a buffering agent on the results of *in vitro* tests from the standpoint of its effect on formation of the hydroxyapatite surface layer.

EXPERIMENTAL PART

The samples employed

The experiments were carried out with glasses of the system $\text{Na}_2\text{O-CaO-SiO}_2$, the same as were employed in the previous study [6]. The compositions of the glasses are listed in table 1. The tests were performed on both compact and ground samples prepared in the same way as in [6].

Table 1. Composition of the glasses.

Glasses	composition (wt.%)			
	SiO ₂	Na ₂ O	CaO	P ₂ O 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 [7].

Exposure of the glasses to model solutions

The basic model solution used in the exposure of samples was the simulated body fluid (SBF) whose composition is close to that of the inorganic part of human blood plasma (table 2). The solution was buffered with TRIS and the *pH* was adjusted with HCl to 7.2–7.3 at 37 °C. Another solution employed was SBF alone free of TRIS, and for the sake of comparison also distilled water with and without TRIS. For assessing the effect of the acid used in adjusting the *pH* of the simulated body fluid, additional model solutions were buffered with TRIS combined with either HCl or citric acid, and for the sake of comparison, an aqueous citrate solution with its *pH* identical with that of SBF with citric acid was prepared.

The samples were extracted under the same conditions as in study [6]: temperature 37 ± 0.4 °C, time of exposure max. 12 days, static conditions of leaching. In the case of ground samples the ratio of glass surface area to the leaching solution volume *S/V* was approximately 1 cm⁻¹; with compact samples it amounted to about 0.13 cm⁻¹.

Table 2. 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

Analyses of the leachates and evaluation of the glass surfaces

The interactions of glasses with the leaching solutions were assessed on the basis of analyses of leachates and on that of studying the changes in the glass surface by X-ray diffraction analysis and IR reflex spectroscopy. The ability to form hydroxyapatite on the glass surface and the rate of its forming were assessed by determining the changes in the concentration of

phosphates and calcium in the solution. According to the results obtained in Part 1 of the present study [6] it may be assumed that the decrements in the content of these components are indicative of formation of a glass surface layer enriched with phosphorus and calcium. At the same time, however, the changes in phosphorus concentration and particularly those in calcium concentration are influenced by solubility of the glass insofar it contains the components. In order to assess the solubility of glasses in the leachates, the concentrations of SiO₂ were likewise determined.

The PO₄³⁻ and SiO₂ concentrations in the leachates were determined spectrophotometrically, and the concentrations of Ca²⁺ by the AAS method.

The surfaces of glasses were analyzed on the Seifert 3000P X-ray diffractometer. The IR spectra were measured by the Nicolet 740 spectrometer using the diffusive reflex method, similarly as in the previous study [6].

RESULTS AND DISCUSSION

The effect of TRIS

Study [2] showed that TRIS promotes leaching of bioactive glass-ceramics and on the basis of this the authors assume that its presence in solution inhibits formation of hydroxyapatite in the surface of these materials. In order to assess more closely the effect of TRIS on the results of tests with bioactive glasses, compact specimens of the glasses were exposed to SBF both with and without TRIS. With all of the types of glass, analyses of the extracts showed substantial differences in the results of leaching into SBF with TRIS and those into SBF free of TRIS (figures 1, 2, 3).

The concentrations of SiO₂ in leachates into SBF containing TRIS were in all instances higher than those in extracts into TRIS-free SBF (figure 1). With all the glasses, the SBF containing TRIS also showed increments in Ca²⁺ concentrations in solution, unlike the case of TRIS-free SBF where no such increments, but on the contrary slight declines of Ca²⁺ concentration were noted (figure 2). All these results bear out the fact that leaching of the glasses is promoted by the presence of TRIS.

For assessing the formation of hydroxyapatite on the surface of glass it is important to know in particular the changes in phosphorus concentration in solution. Following exposure to TRIS-free SBF, lower decrements of PO₄³⁻ concentration in solution were found with phosphorus-free glasses (S45, S55) compared to extraction into TRIS-containing SBF (figure 3). With glasses containing phosphorus and exposed for shorter periods to TRIS-free SBF, the phosphorus content in solution did not increase, unlike the case of TRIS-containing SBF where only losses in the content of phosphates in solution were established.

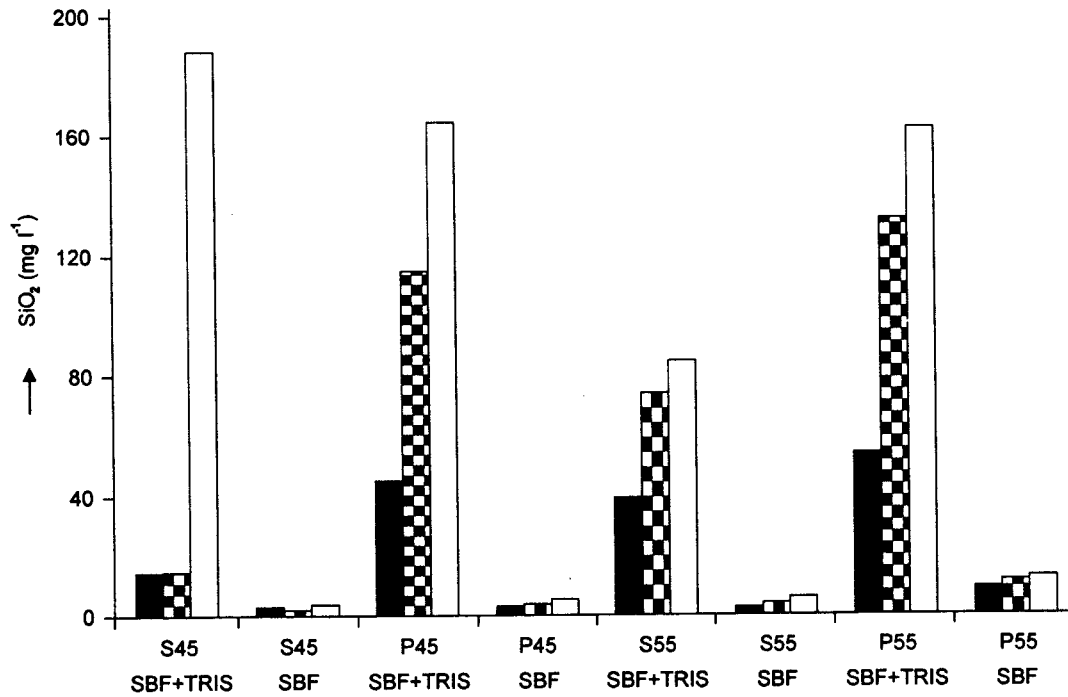


Figure 1. Comparison of SiO₂ concentrations in extracts into SBF with TRIS (SBF + TRIS) and into SBF without TRIS (SBF). Compact samples; $S/V = 0.13 \text{ cm}^{-1}$;
 ■ - 5 hours, ▣ - 24 hours, □ - 120 hours

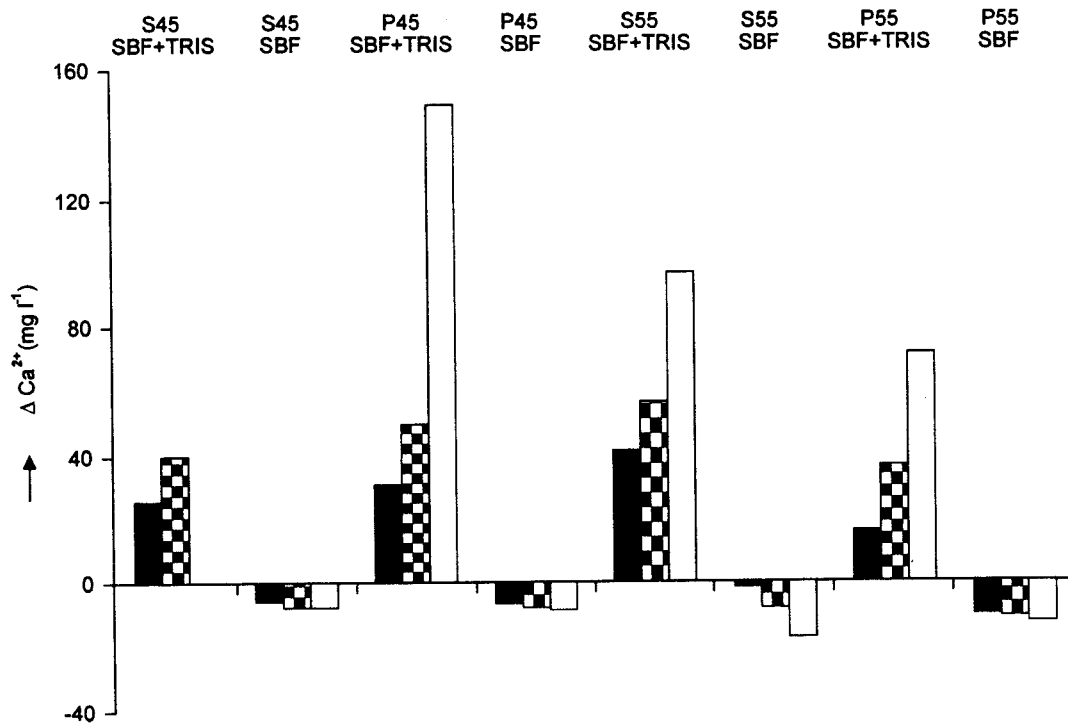


Figure 2. Comparison of changes in Ca²⁺ concentration in extracts into SBF with TRIS, and into SBF without TRIS. Compact samples; S/V about 0.13 cm^{-1} ;
 ■ - 5 hours, ▣ - 24 hours, □ - 120 hours

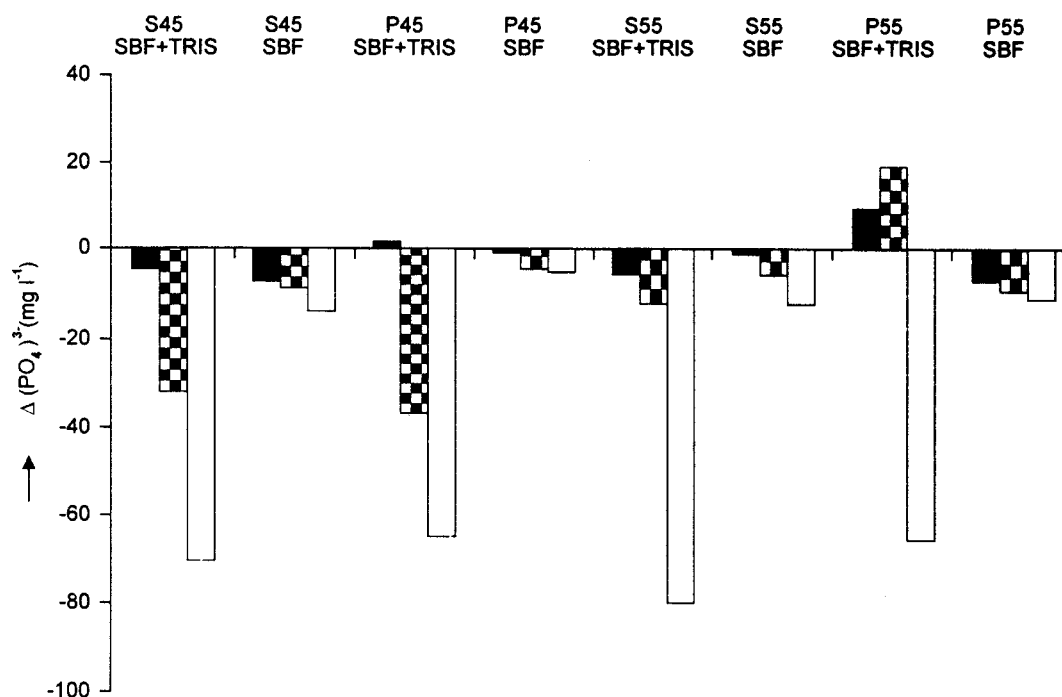


Figure 3. Comparison of changes in PO_4^{3-} concentration in extracts into SBF with TRIS, and into SBF without TRIS. Compact samples; $S/V = 0.13 \text{ cm}^{-1}$;
 ■ - 5 hours, ▣ - 24 hours, □ - 120 hours

Also these results indicate that TRIS speeds up extraction of glasses. In this way, from phosphorus-containing glasses more phosphorus passes into solution with TRIS than into that without TRIS. At the same time, a comparison of changes in phosphorus concentrations in solution for the two types of glass leads to the assumption that TRIS supports formation of a phosphorus-enriched layer on the glass surface.

The conclusions reached on the basis of analyses of extracts were borne out by evaluation of glass surfaces with the use of IR spectroscopy and X-ray diffraction analysis (table 3). Hydroxyapatite was identified by IR spectroscopy in the surface after 24-hour exposure in TRIS-containing SBF of the phosphorus-containing glasses (P45 and P50). The same compound was likewise established in the surface of the glasses by X-ray diffraction analysis after 5-day exposure to SBF with TRIS. On the other hand, no hydroxyapatite was identified in the surface of the same glasses extracted in SBF free of TRIS not even after 5 days of exposure. In the case of phosphorus-free glass (S50), IR spectroscopy also identified hydroxyapatite after 24 hours of exposure to SBF with TRIS, whereas no crystalline phase was found after extraction in TRIS-free SBF.

The results of analyses of extracts and those of assessing the glass surfaces lead to the conclusion that TRIS speeds up considerably the leaching of glasses,

which is in agreement with the results obtained in the study of glass-ceramic materials [2]. However, with the glasses in question TRIS also accelerates formation of the hydroxyapatite layer on the glass surface.

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	IR
P50	SBF+TRIS	24	0	HA
		120	HA	HA
	SBF	120	0	0
		SBF+citric acid	120	0
P45	SBF+TRIS	5	not measured	HA?
		24	not measured	HA
	SBF	5	not measured	0
		24	not measured	0
S50	SBF+TRIS	24	0	HA
	SBF	24	0	0

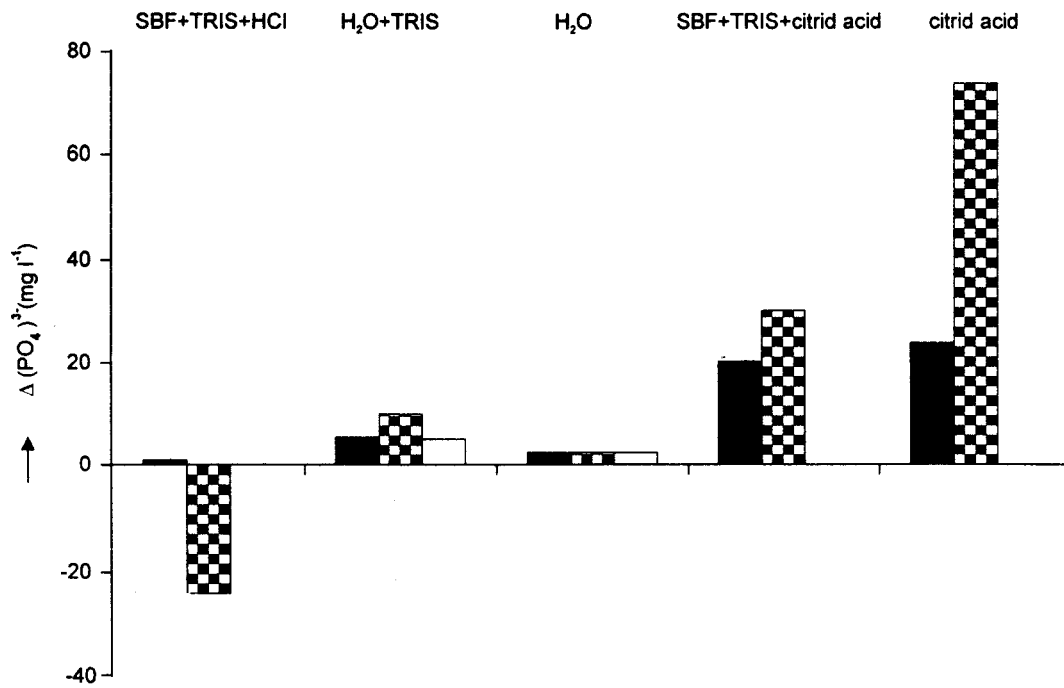


Figure 4. Comparison of changes in PO_4^{3-} concentration in model solutions. Glass P45; ground samples; S/V about 1 cm^{-1} ; ■ - 7 hours, ▣ - 24 hours, □ - 120 hours

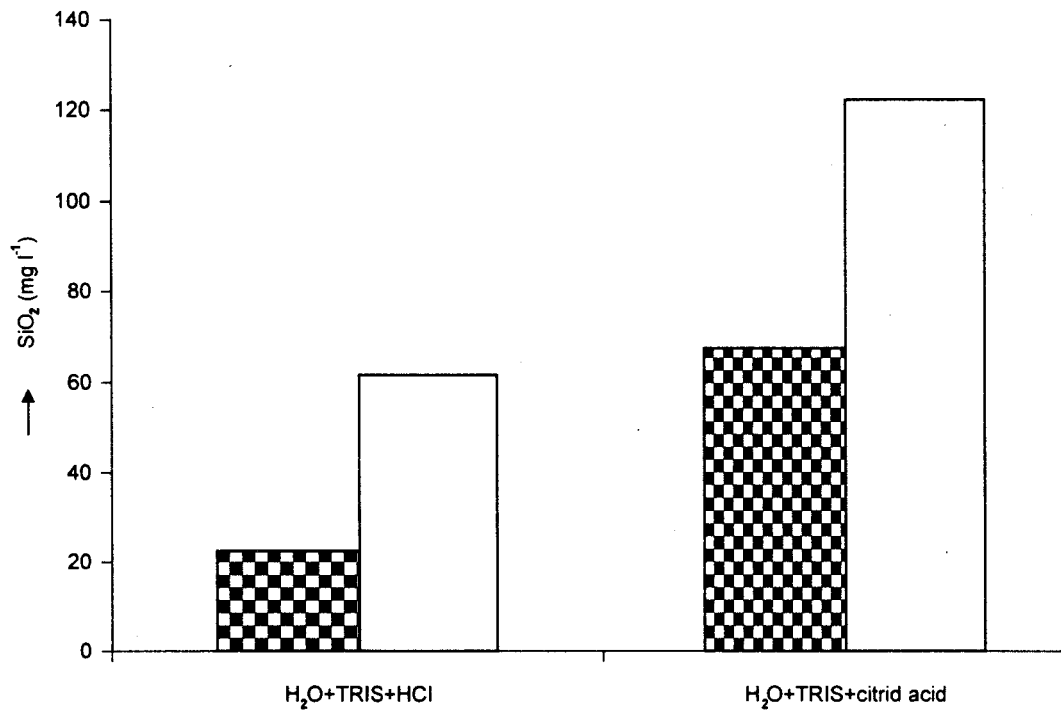


Figure 5. Comparison of changes in SiO_2 concentration in model solutions. Glass P45; ground samples; S/V about 1 cm^{-1} ; ▣ - 7 hours, □ - 24 hours

The effect of citrate

For the purpose of assessing the effect of citrate ions when using citric acid in place of HCl for adjusting the *pH* of SBF solution containing TRIS, the influence of citrate ions in a neutral medium on the results of the tests was investigated. Following 7- and 24-hour exposure of P45 ground glass sample to the SBF + TRIS + citric acid combination, the increase in phosphorus content in solution was higher than with SBF + HCl and no decrease in its content was noted (figure 4). The amount of SiO₂ passed from glass into the SBF solution adjusted with TRIS and HCl was likewise lower than in the case of the aqueous solution with TRIS and citric acid (figure 5). In the case of leachates into SBF buffered with TRIS and HCl the *pH* value remained fairly constant even after 12 days of exposure as compared to the solution buffered with TRIS and citric acid whose *pH* increased significantly (from 7.3 up to 8.3).

The results show that citrate in neutral and alkaline solutions promotes significantly the dissolution of glass, even more than TRIS alone. At the same time they allow to assume that unlike the case of TRIS, the presence of citrate ions in solution inhibits formation of a phosphorus-enriched layer on the glass surface as a result of their accelerating effect on the dissolution of glass. This is also borne out by the results of evaluating the surface by X-ray diffraction and IR spectroscopy in the case of compact sample P50, on the surface of which no hydroxyapatite was identified even after 5 days of exposure to SBF adjusted with TRIS and citric acid, in contrast to SBF combined with TRIS + HCl. The results are in full agreement with the results of [3] according to which no hydroxyapatite is formed on the surface of glasses leached with SBF whose *pH* has been adjusted with TRIS and citrate. The difference in the effect of TRIS alone and that of the TRIS-citrate combination on the formation of hydroxyapatite layer on the surface of glass can obviously be explained by the fact that whereas TRIS forms soluble complexes with calcium ions [2], citrate in neutral medium moreover forms complexes with silicon [4, 5].

TRIS alone does not seem to produce complexes with silicon ions, as indicated by [5], where, contrary to citrate, no accelerating effect of TRIS on dissolution of pure silica glass was observed.

The dissolution of glass in the presence of citrate is obviously so fast that no protective layer enriched with phosphorus and calcium manages to form. In SBF solutions containing only TRIS and HCl and no citrate ions, and where only TRIS is assumed to produce complexes with calcium, the silicate lattice is not impaired to such a significant degree and the glass does not dissolve, so that hydroxyapatite has the time to form

a layer on the surface. According to the results of the present study, in this case the presence of TRIS in fact speeds up creation of the hydroxyapatite surface layer. In study [2] it was found that an accelerating effect on extraction of Ca²⁺ ions from glass-ceramic materials into SBF, similar to that of TRIS, is also exhibited by albumin. It cannot be ruled out that TRIS in SBF simulates to a certain degree the effect of some organic components in the body fluid.

CONCLUSION

The organic compounds used in buffering model solutions when assessing the suitability of bioactive materials by *in vitro* testing may influence the test results. The presence of the most frequently employed TRIS buffer (in combination with HCl) speeds up significantly the leaching of glasses in the system Na₂O-CaO-SiO₂-P₂O₅, while at the same time promoting formation of the hydroxyapatite layer on the glass surface.

The presence of citrate ions in solution with TRIS (the TRIS/citric acid combination) accelerates the extraction as well as dissolution of glass to a greater degree than TRIS alone. However, in contrast to TRIS alone (solely in combination with HCl), in the presence of citrate ions the formation of a surface layer enriched with calcium and phosphorus is suppressed.

The difference in the effects of the two agents is obviously due to the fact that TRIS produces soluble complexes solely with calcium ions, whereas citrate in neutral and alkaline medium is assumed to form complexes with both calcium ions and silicon. This brings about degradation of the silicate glass lattice and its dissolution that proceeds at such a high rate that the hydroxyapatite surface layer is not able to form.

Acknowledgement

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STUDIUM BIOAKTIVNÍCH SKEL TESTY "IN VITRO"

ČÁST 2 – VLIV PUFRU TRIS NA VÝSLEDKY TESTŮ

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Před vlastní aplikací biomateriálu do lidského organismu, jsou sledovány jeho vlastnosti testy *in vivo*, tzn. v živém organismu zvířete a testy *in vitro*, tzn. v prostředí simulujícím tělní tekutinu.

Při testech *in vitro*, zejména u dlouhodobých experimentů, je důležité, aby se pH modelových roztoků neměnilo, proto jsou loužící roztoky pufrovány. Organické sloučeniny používané k pufrování modelových roztoků mohou ale ovlivnit výsledky testů. Cílem předložené práce bylo posoudit vliv nejčastěji používaného pufru TRIS na výsledky testů *in vitro* z hlediska jeho vlivu na tvorbu povrchové vrstvy hydroxyapatitu.

Pro podrobnější posouzení vlivu TRISu na výsledky testů u bioaktivních skel byly exponovány celistvé vzorky skel (tab.1) v SBF s TRISEm a bez TRISu. U všech typů skel byly při analýze výluhů zaznamenány výrazné rozdíly mezi výsledky loužení do SBF s TRISEm a SBF bez TRISu (obr.1, 2, 3). Koncentrace SiO_2 ve výlužích do SBF s TRISEm byly u všech skel výrazně vyšší než u SBF bez TRISu (obr.1). U všech skel byly dále zaznamenány v SBF s TRISEm přírůstky Ca^{2+} v roztoku, zatímco u SBF bez TRISu přírůstky nalezeny nebyly, naopak pouze malé úbytky Ca^{2+} (obr.2).

Pro posouzení tvorby hydroxyapatitu na povrchu skla jsou důležité především změny koncentrace fosforu v roztoku (obr.3). U skel obsahujících fosfor přechází ze skla do roztoku s TRISEm více fosforu, než do roztoku bez TRISu. Všechny tyto výsledky potvrzují, že TRIS urychluje loužení skel. Porovnání změn koncentrace fosforu v roztoku u obou typů skel (s obsahem i bez obsahu fosforu) současně vede k předpokladu, že TRIS podporuje tvorbu vrstvy obohacené o fosfor v povrchu skla.

Tento předpoklad byl potvrzen hodnocením povrchů skel IČ spektroskopii a RTG difrakční analýzou, kdy u vzorků skel loužených v SBF s TRISEm byl identifikován hydroxyapatit po kratší době expozice, než u skel loužených v SBF bez TRISu.

Hodnota pH se u SBF s TRISEm na požadovanou hodnotu nejčastěji upravuje pomocí HCl. Pro posouzení vlivu citrátových iontů při použití kyseliny citronové místo HCl při úpravě pH byl dále sledován vliv citrátových iontů na výsledky testů.

U drťového vzorku skla P45 byl po expozicích 7 a 24 h v SBF s kombinací TRIS+kyselina citronová naměřen vyšší nárůst obsahu fosforu v roztoku oproti TRISu s HCl a nebyl zde zaznamenán jeho pokles (obr.4). Obsah SiO_2 přešlého ze skla do výluhu byl u vodného roztoku TRISu s HCl rovněž nižší, než u vodného roztoku TRISu s kyselinou citronovou (obr.5). Uvedené výsledky potvrzují, že citrát v neutrálních a alkalických roztocích výrazně urychluje rozpouštění skla a to více, než samotný TRIS. Současně z nich lze usoudit, že rozpouštění skla je v přítomnosti citrátu zřejmě natolik rychlé, že se nestačí vytvářet povrchová vrstva obohacená o fosfor a vápník. Tento předpoklad opět potvrdily výsledky hodnocení povrchu skel IČ spektroskopii a RTG difrakční analýzou, kdy v povrchu vzorků loužených za přítomnosti citrátových iontů nebyl identifikován hydroxyapatit.