

MECHANISM OF BIOACTIVITY OF LS2-FA GLASS-CERAMICS IN SBF AND DMEM MEDIUM

[#]GABRIELA LUTIŠANOVÁ, MARTIN T. PALOU, JANA KOZÁNKOVÁ

Institute of Inorganic Chemistry, Technology and Materials, Faculty of Chemical and Food Technology, Slovak University of Technology in Bratislava, Radlinského 9, 812 37 Bratislava, Slovakia

[#]E-mail: gabriela.lutisanova@stuba.sk

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In this paper, the results of in vitro bioactivity of glass-ceramics in the $\text{Li}_2\text{O-SiO}_2\text{-CaO-P}_2\text{O}_5\text{-CaF}_2$ (shorthand LS2-FA) system immersed in two media (simulated body fluid (SBF) and Dulbecco's modified Eagle's medium (DMEM)) are compared. Microprobe (EPMA) and Scanning Electron Microscopy (SEM) were used to detect the presence of a new phase on the surface and to characterize its layer. Inductively coupled plasma (ICP) was used to monitor ion concentration changes in SBF with immersion time. The results show, that during the assay in vitro behaviour tests, the surface of the sample was partially dissolved and released of Si^{4+} , Ca^{2+} and Li^+ ions were released into the SBF medium. The results of surface characterization after in vitro tests revealed difference in the bioactivity of glass-ceramics with various time of immersion in SBF and DMEM. For the formation of an apatite-layer in an earlier testing period (1, 3 and 7 days), a pronounced difference was not observed between SBF and DMEM immersion. In the longer testing period in SBF (28 days), the apatite-layer was developed by periodic deposition of spherical bullets that covers the whole surface. The use of the acellular culture medium DMEM resulted in a delay at the start of precipitation.

INTRODUCTION

$\text{Li}_2\text{O-SiO}_2$ systems with composition close to lithium disilicate (LS2) are ones of the most studied systems regarding the control crystallization in glass-ceramic synthesis. Stookey (1959) developed the first glass-ceramics by heat treatment of glass from this system [1]. The research based on LS2 glass-ceramics can be classified into two categories. The first one deals with the study of a binary system. Study is focused on the nucleation mechanism and identification of the primary phases, which prevent the lithium disilicate precipitation [2, 3, 4]. The second field deals with the multicomponent systems. In this case some oxides such as MgO , Al_2O_3 , B_2O_3 , Fe_2O_3 , P_2O_5 , ZnO , and TiO_2 , are added to binary $\text{Li}_2\text{O-SiO}_2$ systems in order to develop new glass-ceramic materials designed especially for biomedical applications. Many authors [5-11] have shown that thermal, optical and mechanical properties of these materials, deduced from their microstructure and phase composition, can be obtained by controlled crystallization and grain growth processes. Therefore many of these glass-ceramic materials can be used as biomaterials - implants in two various departments: First, they may be used as highly durable materials in restorative dentistry. Second, they may be applied as bioactive materials for the replacement of hard tissues in living body [2, 12-15].

After implantation of biomaterials in living tissues calcium phosphate deposition and protein adsorption are supposed to appear on their surfaces, thus proving their bioactivity. The formation of calcium phosphate layers (apatite) detected in synthetic solutions containing calcium and phosphate ions, is one of the methods used to investigate and/or activate bioactivity in synthesized materials [16-19]. The description of these new layers and the kinetics of their formation depend on the composition used of the synthetic simulated solution. Synthetic fluids like SBF and DMEM have a few compositional distinction when compared with the inorganic fraction of the human blood plasma, especially Cl^- and lower HCO_3^- concentrations and, in the case of SBF, the presence of a nonphysiological buffer Tris. Bohner and Lemaitre (2009) in their research mentioned the ambiguity between the *in vitro* tests that can produce false positive and false negative results and the behavior of implants during cell or *in vivo* tests. The *in vitro* bioactivity test of glasses gives an insight to glass dissolution, pH changes and apatite formation in buffered media with ionic concentration of apatite is of interest for dental applications [20]. The mechanism of the apatite layer nucleation and growth was proposed by Kokubo (1992). It consists of the interchange between Ca^{2+} ions of the glass and the H_3O^+ of the solution and then it forms Si-OH groups on the glass surface, activating apatite

nucleation [21]. The nuclei thus formed grow at the expense of the ions in the solution saturated with respect to the apatite [22, 23].

In this context, several studies of *in vitro* bioactivity have been investigated for glasses belonging to various systems, for example CaO–P₂O₅–SiO₂ [22, 24–27], CaO–MgO–P₂O₅–SiO₂ [28, 29] and CaO–SiO₂ [30]. It was revealed that the immersion of the glass in SBF led to an increase of Ca²⁺ concentration and pH due to a partial dissolution of glass network. Müller and Müller (2006) stressed that some conditions of the *in vitro* experiments, such as temperature, composition and pH of the leaching solution, the time and nature of exposure (static or dynamic conditions) and the ratio of the material's surface area to the volume of the leaching solution (S/V) are necessary to observe precisely. Otherwise, the results can be significantly influenced when changing just one of the conditions described above [31].

The objective of the present study was to synthesize glasses and glass-ceramic derivatives in the system Li₂O–SiO₂ with the different contents of CaO, P₂O₅ and CaF₂ in relative ratios with respect to fluorapatite (FA). FA which is known as bioactive material has been added to initial lithium disilicate composition. *In vitro* bioactivity of the glass-ceramic samples obtained from glass system was evaluated by monitoring the pH changes, the concentration of calcium, silicon and phosphorous in SBF medium, measurements of solubility and by analyzing newly created surface layer and its thickness with immersion time in SBF and DMEM media by SEM and EPMA techniques.

EXPERIMENTAL

Samples preparation

As starting powders, Li₂CO₃ (≥ 98 wt. %, Fluka, USA), ground quartz sand (SiO₂, 99.6 wt. % SiO₂), dried CaF₂ (99.9 wt. %, Sigma-Aldrich, USA) and Ca₃(PO₄)₂ (96 %, Fluka, USA) were used. The weight percentages are presented in Table 1. The ratio of CaF₂ and Ca₃(PO₄)₂ is kept to stoichiometric FA composition. A powder mixture was melted in Pt crucible in a supercanthal furnace at 1450°C (2 h, 10°C/min) with intermediate grinding and with a calcination step (5 h at 950°C). Then the melts were quenched by pouring them onto a copper board and then placed in heated muffle furnace at 450°C. The muffle was switched out and glass samples were slowly cooled to ambient temperature. Such prepared glass samples were crushed into powder, homogenized, remelted and poured into copper moulds to form discs with precisely defined dimensions as listed above.

The representative glass-ceramic samples with 33.00 wt. % FA (14 wt. % P₂O₅) were synthesized by annealing or thermal treating of parent glasses (6 h at 600°C, heating rate 10°C min⁻¹) as reported [32] to characterize the crystallization course of different phases.

Table 1. The chemical composition of the investigated materials in wt. %.

Components	FA content (wt. %)
	33.15
SiO ₂	72.87
Li ₂ CO ₃	13.51
CaF ₂	1.05
Ca ₃ (PO ₄) ₂	12.57

In vitro bioactivity

The assessment of *in vitro* bioactivity was carried out by testing in static regime at 36.5°C for the following time intervals: 1, 3, 7 and 28 days using two biological media – SBF and DMEM. Carefully cleaned samples were poured in the plastic containers in a volume of model solution corresponding to S_a/V_s ≅ 10 cm⁻¹ (Equation 1) [19] and heated up to the temperature of 36.5°C. After exposure in media, samples were taken out and washed gently with ethanol and distilled water. Then the samples were left to dry at ambient temperature inside the desiccator for further analysis.

The SBF is an acellular, aqueous medium with an ionic composition which resembles that of human blood plasma (Table 2). This medium was prepared by dissolving NaCl, NaHCO₃, KCl, K₂HPO₄·3H₂O, MgCl₂·6H₂O, CaCl₂·6H₂O, Na₂SO₄ per litre of ultra pure water in a beaker. The pH was adjusted using 1 M hydrochloric acid according to the prescription developed by Kokubo *et al.* [33]. Sodium azide (NaN₃) was added to inhibit bacterial growth. The medium was kept at 36.5°C in an incubation apparatus. The microbiological assay of SBF medium indicated the necessity of NaN₃ addition to inhibit the growth of bacteria. Gil stated that the use of SBF without NaN₃ addition led to an enormous increase of cells (10⁴-10⁵ /ml) in the form of coliform bacterium, bacterium bacillus and Pseudomonas after cultivation in culture media at 36.5°C. These anaerobic bacteria consume phosphorus and thus reduce its concentration

Table 2. Ion concentrations of SBF and DMEM medium in comparison with those in human blood plasma [23].

Ion	Ion concentrations (mmol l ⁻¹)		
	Blood plasma	SBF	DMEM
Na ⁺	142.0	141.8	154.56
K ⁺	3.6-5.5	5.0	5.37
Mg ²⁺	1.0	1.5	0.8
Ca ²⁺	2.1-2.6	2.5	1.82
Cl ⁻	95.0-107.0	148.0	120.5
HCO ₃ ⁻	27.0	4.2	44.0
HPO ₄ ²⁻	0.65-1.45	1.0	1.0
SO ₄ ²⁻	1.0	0.5	0.8
pH	7.25-7.4	7.4	7.3

in the medium. Furthermore, it was observed that these bacteria may have a negative impact on human health [34].

DMEM is a conventional acellular fluid (Sigma-Aldrich, Germany), which in addition to containing similar ionic concentrations as blood plasma (Table 2), also contains growth factors, proteins, hormones and vitamins common to blood.

All samples before immersion in both media were cleaned with the UV treatment and rinsed with ethanol and distilled water. After exposure in both media, samples were taken out and washed gently with distilled water. Then the samples were left to dry for further analysis at ambient temperature inside a desiccator.

EXPERIMENTAL METHODS

Leachate analysis and solubility measurements of samples

The solutions of the bioactivity behaviour tests were analyzed using induced coupled plasma (ICP-OES) spectroscopy in order to determine the elemental concentration of Li^+ , Si^{4+} , Ca^{2+} and P^{5+} as function of immersion time. At the time of solubility measurements, the pH of the solution was also measured as a function of time employing a pH meter with accuracy of 0.01. The pH was measured using a pH meter inoLab pH 730 with electrode WTW Sen Tix HW. The percentage of error in the measurement of pH is ± 0.005 . These results are very useful for an understanding of the phenomenon of ion transfer which can take place between the glass-ceramic surface and the simulated body fluid. The solubility of glass-ceramic samples was evaluated by the measurement of weight loss in simulated body fluid (SBF) at 36.5°C in the incubator apparatus. Solubility can directly be correlated to sample corrosion. Samples were washed ultrasonically in acetone for a minute and were placed in polyethylene bottles in a volume of SBF corresponding to equation (1). At different points of time - 0, 1, 12, 24, 72, 168, 468 and 672 h, the samples were taken out from the SBF and excess moisture was eliminated within in 24 h drying at an ambient temperature in the desiccator. Then the samples were weighed. The change in weight loss can be directly correlated to glass-ceramic corrosion or solubility in SBF. The amount of weight loss was calculated using the following relation:

$$wt_{loss} = (w_0 - w_t)/w_0 \quad (2)$$

where w_0 is the initial weight of the specimen and w_t the weight at each period of time. The measurements were carried out in duplicate.

Surface analysis

Surface microstructure and apatite-layer formation on samples before and after immersion in SBF and

DMEM media were studied by SEM (TESLA BS 300 with digital unit TESCAN). The samples were sputtered coated with Au. The Microprobe (EPMA JEOL JXA-840A) was used to examine the surface layer formed on the samples during exposure in SBF and DMEM media. Samples were carbon-coated before analysis.

RESULTS AND DISCUSSION

Analysis of leachates

During the bioactivity tests of LS2-FA samples, the SBF solutions were analyzed using induced coupled plasma (ICP-OES) spectroscopy in order to determine the elemental concentration of Ca^{2+} , Si^{4+} , P^{5+} and Li^+ ions as a function of immersion time (Figure 1). Every data point in the figures was obtained as the average of two values. For comparison, the values of these ion concentrations in human plasma were also included in the figure according [35]. ICP measurements showed the changes of ion concentration in SBF with time of immersion and primarily due to the formation of apatite. Changes in the ionic concentration of the SBF medium can be interpreted as a series of reactions occurring on the surface of the glass-ceramic composites sample. These results are very useful for understanding the phenomenon of ion transfer which takes place between the glass-ceramic surface and the synthetic physiological liquid.

It can be seen that the curve of the calcium concentration increased with time of immersion in SBF, indicating the dissolution of the calcium-containing phase of the sample. The rapid release of Ca^{2+} ions into the SBF medium (from 2.5 mmol.l^{-1} to 5.3 mmol.l^{-1}), as well as the pH increase (see Figure 1) during the 168 hours of the assay, are due to partial dissolution of the surface. After this period, the Ca^{2+} concentration did not change significantly. Between the 20th and the 28th day we observe the stabilization of leach.

The decrease in the phosphorus concentrations is likely due to the precipitation of the calcium phosphate (Ca-P) layer, which consumes Ca^{2+} and P^{5+} ions from the SBF medium [36, 37]. The formation of the Ca-P layer on the surface of samples is the main indicator of bioactivity. Changes in the Ca^{2+} concentration correlated with changes in the PO_4^{3-} concentration confirm the precipitation of calcium phosphate.

As can be seen in Figure 1, silicon concentration released into the SBF medium increases steadily with time of immersion. The increase in Si^{4+} indicates the first stage of dissolution by the breaking up of the outer silica layers of the network. The solid silica dissolves in the form of monosilicic acid Si(OH)_4 to the solution resulting from breakage of Si-O-Si bonds and formation of Si-OH (silanols) groups, which act as nucleation sites of the apatite layer. This dissolution mechanism was described by many researchers, such as [36-38].

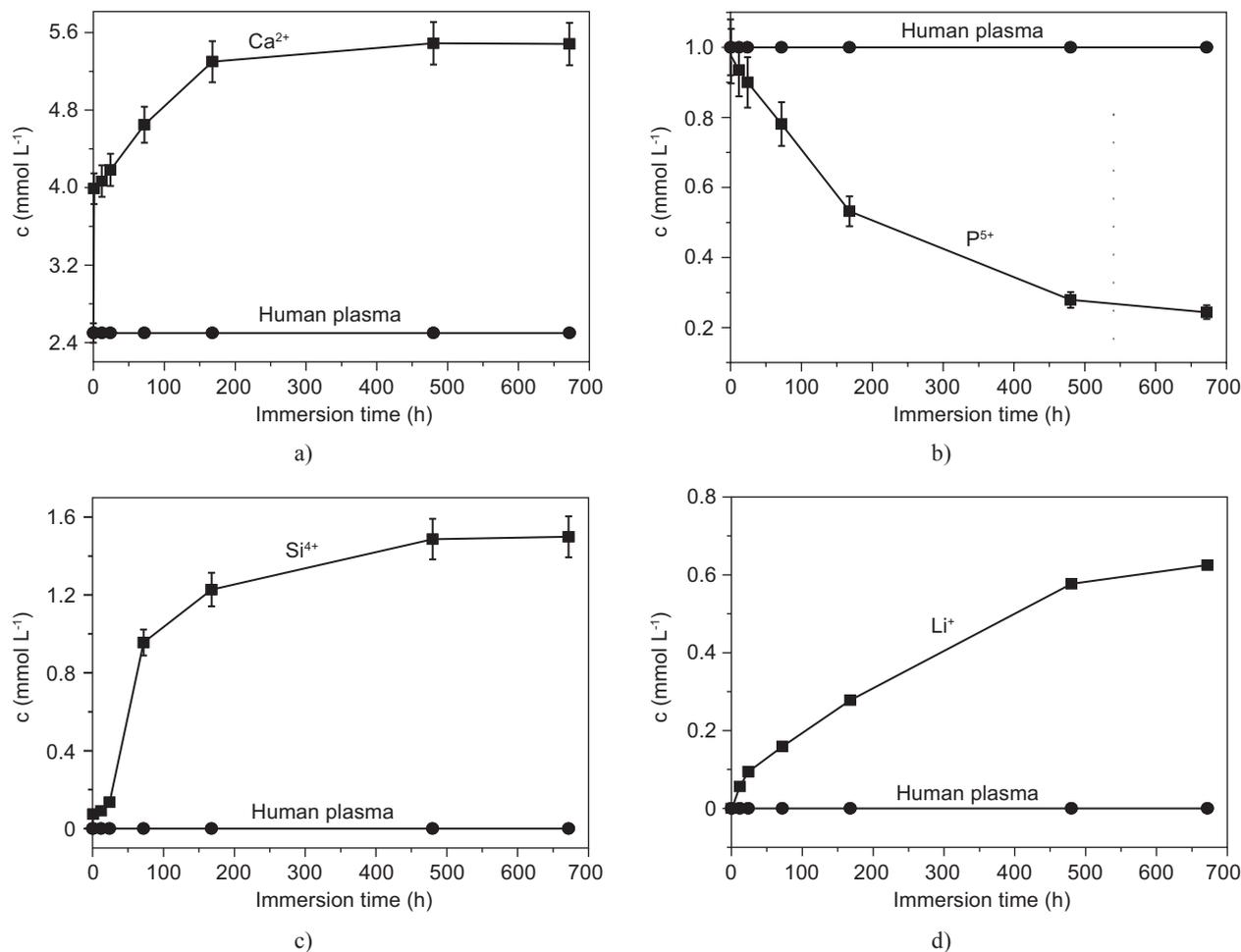


Figure 1. Concentration of Ca^{2+} , P^{5+} , Si^{4+} and Li^{+} leachates with immersion time in SBF.

It is also noted that leachates of lithium concentrations in SBF solution were observed. It is evident that this trend is related to the dissolution of the glass-ceramic phase and the release of Li^{+} ions into the solution, as in cases Ca^{2+} , Si^{4+} , P^{5+} ions. In the first 20 days of immersion can be observed rapidly increase of Li^{+} ions in the SBF solution. After 20 days it tends to be stabilized. The maximum value of Li^{+} concentrations measured in SBF solution after 28 days was 0.667 mmol/l. This value was only slightly increased from the 0.625 which was measured after 20 days of leaching. In contrast with the literature [39, 40], where a level of Li^{+} ions of 0.4-0.8 mmol/l is considered as typical, our measured values are within the tolerance and the samples are not toxic for the human body environment.

Simultaneously, one can observe that the pH increased from the initial value of 7.40 up to 8.46 during 28 day immersion in SBF (Figure 2). The rapid increase in pH in the first stage (between 1 and 7 days) is due to the leaching of cations out of the glass-ceramic samples, which are further exchanged with H^{+} ions from the solution. Hench [36] confirmed that the ion exchange process leads to an increase in interfacial pH with time of immersion, to values >7.4 . In later stage

of the pH measurement (between 7 and 28 days), the pH value increased very slowly in comparison with the first stage resulting in the formation of hydroxyapatite layer on the surface of the samples.

The weight loss of prepared LS2-FA samples immersed in simulated body fluid at 36.5°C as a function of time is shown in Figure 3. The weight loss was presumed to be usually due to ion release and network dissolution

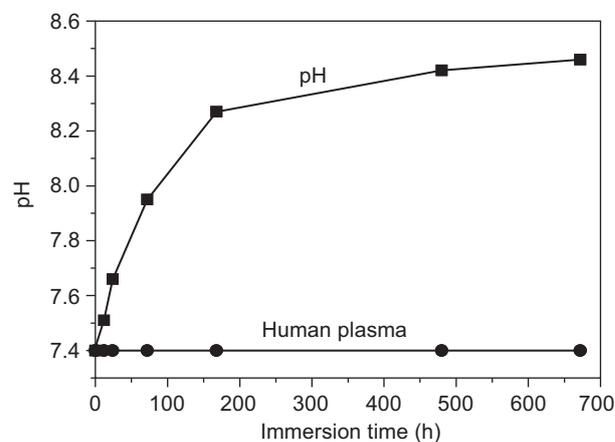


Figure 2. Variation of pH with immersion time in SBF.

of samples by damage of Si-O-Si bonds due to the action of OH⁻ ions. Thus, the dissolution could be monitored by increases of the Si⁴⁺ concentration in the solution and at the same time by weight changes of the samples. High dissolution correlated with high weight loss. However, with the reaction of the glass-ceramic samples in SBF, the weight loss was controlled by the diffusion of relevant ions through the reaction layers.

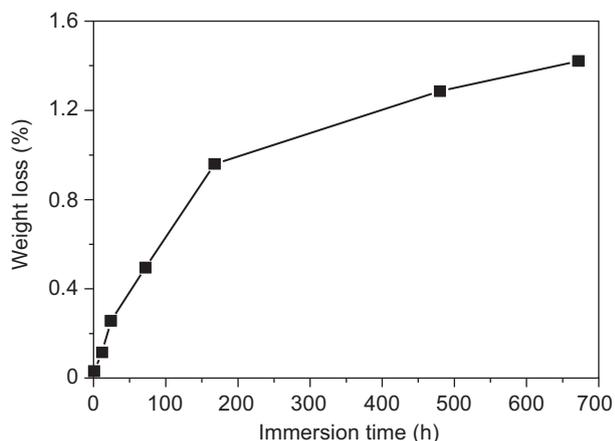


Figure 3. Weight loss as a function of immersion time in SBF.

With the formation of a Ca-P rich layer on the surface, the weight of the glass increased, so the glass-ceramics showed only a moderate weight loss. The weight loss corresponded with the change in pH in the SBF medium. This shows that the dissolution of the samples had a rate equal to the leaching of the ions responsible for the pH increase. The solubility of synthesized materials that can be used as implants plays an important role and significantly affects its stability *in vivo*. Resorption or biodegradation of such materials *in vivo* is a very complicated process, where physiochemical mechanisms interact with the biological mechanisms in protein and

cell-mediated processes. Therefore, it is not exclusively controlled by its physiochemical properties, such as solubility.

In vitro behavior of glass-ceramics

The Scanning Electron Microscopy (SEM) provides insight on the surface microstructure of samples before immersion and the detailed mechanism of apatite-layer formation on the surface of prepared glass-ceramic materials after immersion in biological media. On the surface of LS2-FA glass-ceramics before immersion in both media we can see that the surface is looser and rougher, with holes with irregular shapes and pores (Figure 4a). In their study, Ray and co-workers indicated that these anomalies are due to thermal annealing temperature [41]. The related results from Microprobe (EPMA) analysis (Figure 4b) shows that the surface sample before immersion in synthetic medium is characterized by a presence of silicon with calcium and phosphate originating from mixture of CaF₂ and Ca₃(PO₄)₂ with stoichiometric ratio corresponding to FA (see Table 1).

Figure 5 shows the SEM image of surface microstructure and corresponding apatite-layer thickness of LS2-FA glass-ceramics after 1, 3, 7 and 28 days of assay in SBF without refreshing the medium. As it can be seen from this picture, the sample demonstrated the bioactivity behavior for continuous immersion in SBF medium. Surface microstructure has changed compared with their original form and the thickness of a newly formed surface layer is strongly influenced and changed with time of immersion in SBF.

In earlier testing period - 1, 3 and 7 days in SBF (Figure 5 a, b, c) the sample shows a surface only partially covered with dispersed zones of new phases. EPMA analysis demonstrated that the layer formed on the surfaces is characterized by the presence of silicon, calcium and phosphorus (Figure 6 a, b, c). This evidence

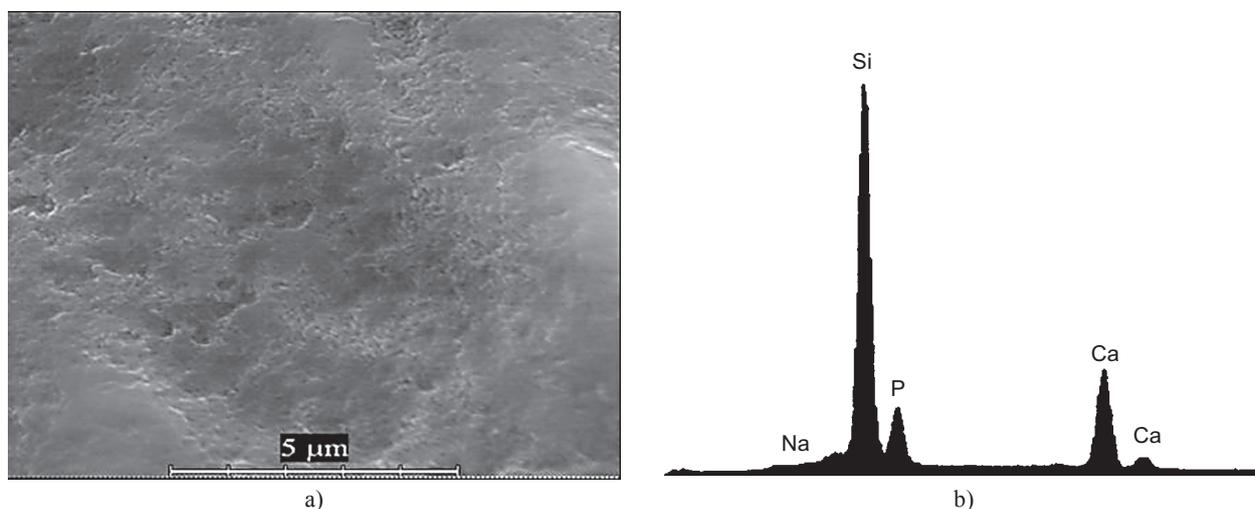


Figure 4. Surface microstructure (a) and EPMA spectra (b) of LS2-FA bioactive glass-ceramics before assay in SBF.

confirms the formation of an apatite-layer on the surface of LS2-FA glass-ceramics. The thickness of a new layer created on the surface varies between 980 nm to 7 μm (see Figure 5 A, B, C).

In the later testing period - 28 days in SBF medium (Figure 5d) we can observe an absolutely dense surface microstructure. The calcium-phosphate (Ca-P) layer was more pronounced, thicker and developed by regular storage of a small spherical apatite bullets that covers the entire glass ceramic surface. The distribution of silicon, phosphorus, and calcium from EPMA analysis differs between the surface glass ceramics (Figure 4b) and the newly formed layer (Figure 6d): The removal of Si and a extremely higher concentration of Ca and P

were observed in the newly formed layer compared to the surface of samples before soaking in SBF medium (Table 3). Except these dominant elements, the presence of small amount of Na, Cl and Mg elements were detected. The above components were derived from SBF. The thickness of Ca-P rich layer created on the surface was about 23.0 μm (Figure 5).

A significant difference of the apatite-layer formed in an earlier testing period - 1, 3 and 7 days - was observed between glass-ceramic samples immersed in SBF and DMEM media. In the case of the glass-ceramic samples soaked in DMEM media one can observe the structural difference which took place on the surface and other globular aggregates with various sizes on the surface (approximately between 680 nm – 3.3 μm). After the immersion of LS2-FA for 1 day in DMEM

Table 3. EPMA analysis of LS2-FA samples after various time immersion in SBF.

Element	Measured content (atomic %) after various time in SBF immersion				
	before immersion	1-day	3-day	7-day	28-day
Si	29.49	27.35	24.75	12.04	–
Ca	2.04	9.16	8.43	22.18	24.36
P	1.29	1.93	2.97	14.13	13.55
Ca/P	1.58	4.75	2.84	1.57	1.80

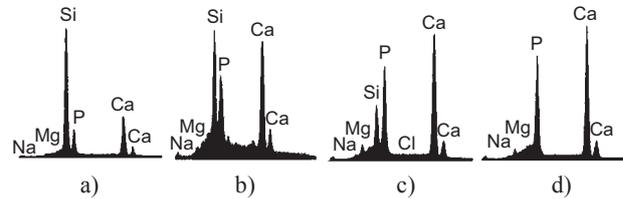


Figure 6. EPMA spectra of LS2-FA samples after: a) 1-day, b) 3-day, c) 7-day and d) 28-day immersion in a SBF medium.

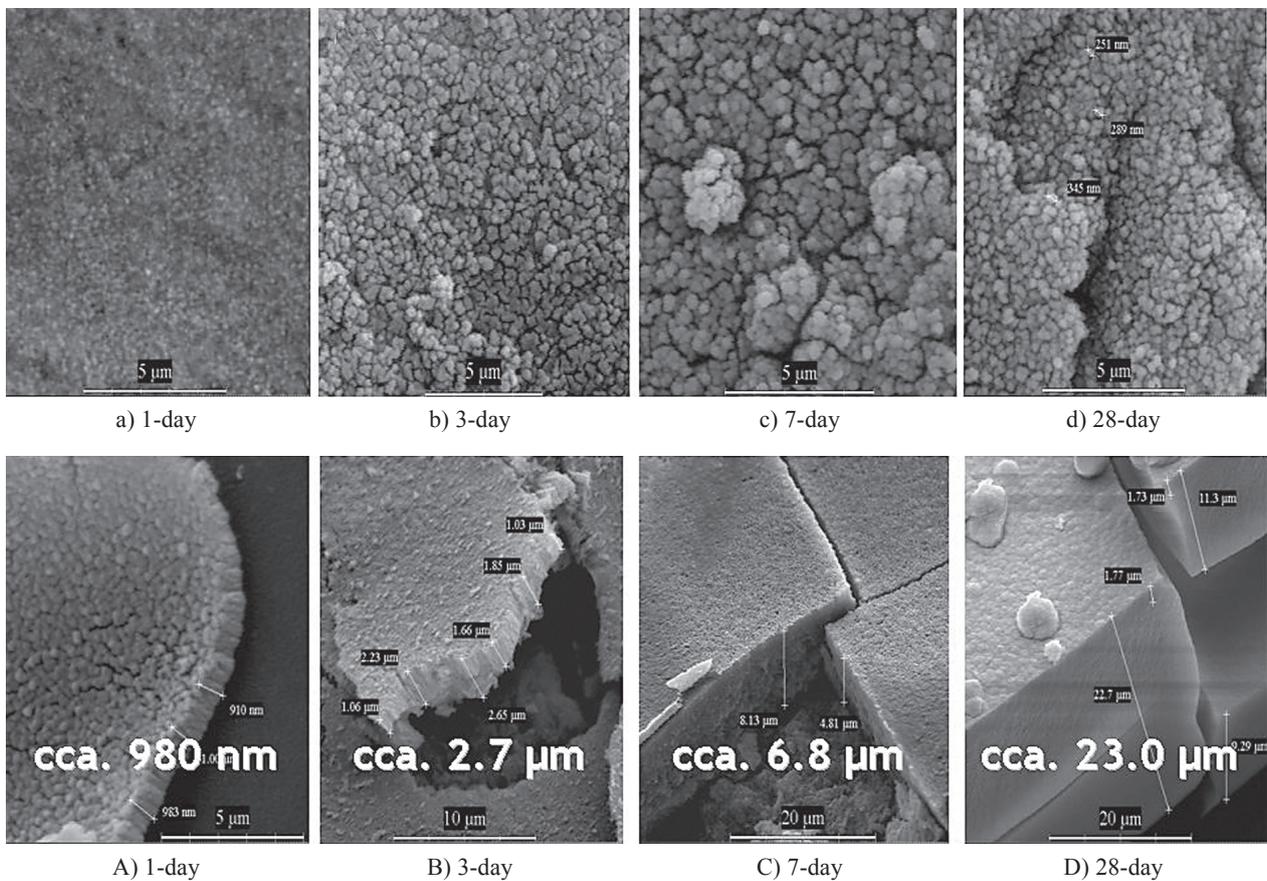


Figure 5. Surface microstructure (a, b, c, d) and corresponding apatite-layer thickness (A, B, C, D) of LS2-FA samples after 1, 3, 7 and 28 day immersion in a SBF medium.

medium, it is possible to observe on their surface the formation of agglomerates formed essentially of silicon and a small presence of calcium (Figure 7 a) with a non-regular morphology. After 3 and 7 days, the surface is partially covered with spherical particles and the overall morphology becomes more regular, and it is possible to distinguish two characteristic morphologies on the surfaces: small particles (spherulitic shape, around 500 nm), and larger crystals (lath shape, around 1.5 μm). SEM and EPMA analysis shows two distinct superimposed layers: on the outermost one can be observed spherical particles composed mainly of Ca and P, whereas the internal layer turns out to be composed essentially of Si (Figure 8).

Table 4. EPMA analysis of LS2-FA samples after various time immersion in DMEM.

Element	Measured content (atomic %) after various time in DMEM immersion				
	before immersion	1-day	3-day	7-day	28-day
Si	29.49	26.17	25.50	11.36	5.99
Ca	2.04	8.04	7.95	16.24	20.99
P	1.29	1.08	2.17	12.25	10.69
Ca/P	1.58	7.45	3.66	1.33	1.96

The later testing period - 28 days in DMEM medium - showed a similar process of apatite-layer formation as in SBF, i.e. with increasing P₂O₅ content in the samples and a longer period of immersion, the thickness of the newly-formed layers increases. But the mechanism of the bioactivity behaviour was slightly slower in this acellular culture medium.

EPMA analysis (Figure 8d) confirmed that the surface appears uniformly covered by spherical particles composed of Ca and P (silicon is also present in a small amounts). The Ca/P ratio (determined by rationing the peaks area derived from semi-quantitative EPMA analysis) for this sample is ~1.96 (Table 4). The thickness reached a value around 7.00 μm (Figure 7 D). The use of acellular culture medium DMEM resulted in a delay at

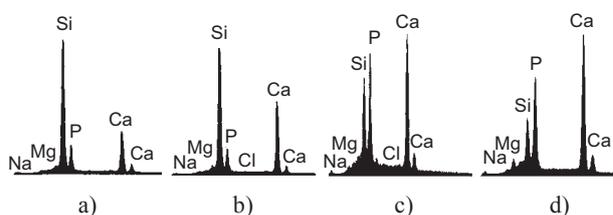


Figure 8. EPMA spectra of LS2-FA glass-ceramic samples after: a) 1-day, b) 3-day, c) 7-day and d) 28-day immersion in DMEM medium.

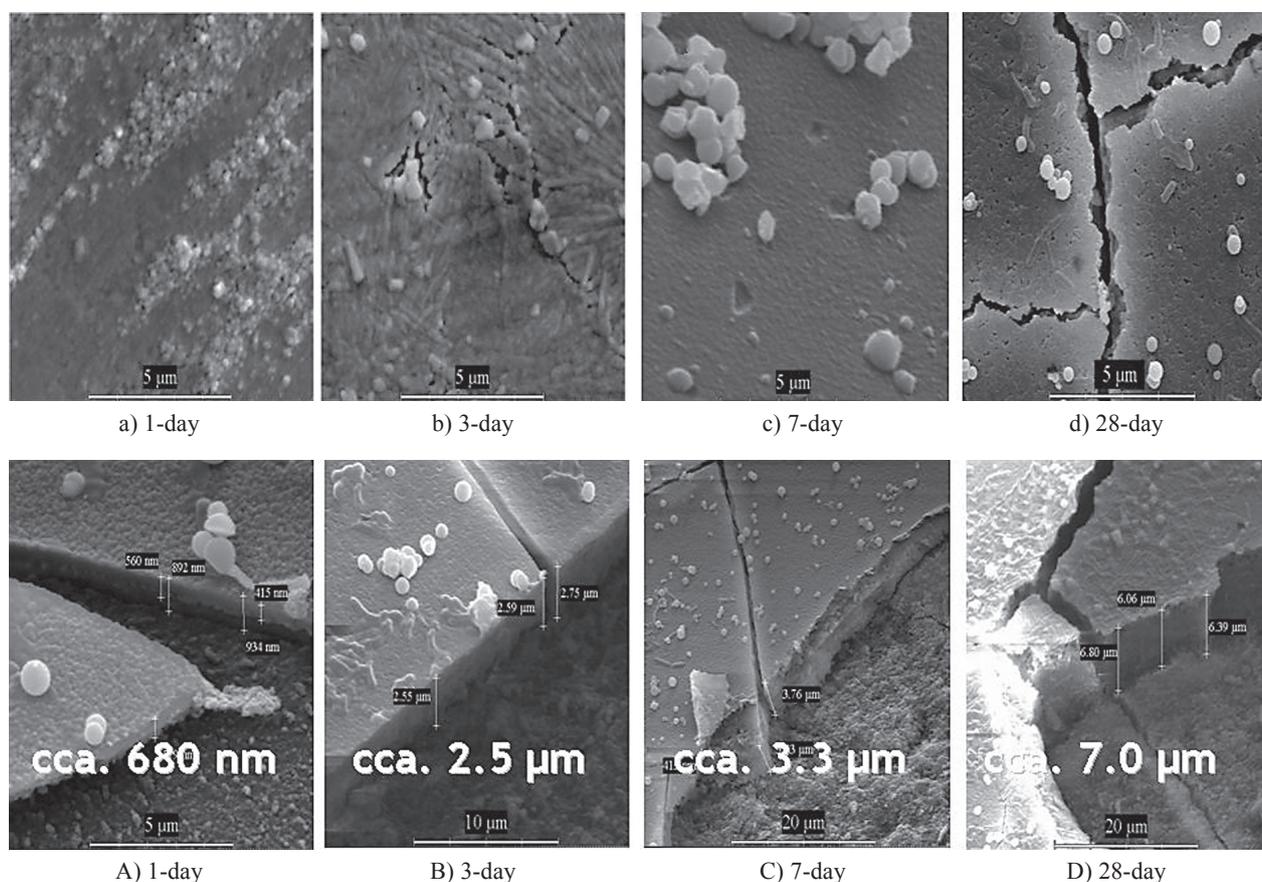


Figure 7. Surface microstructure (a, b, c, d) and corresponding apatite-layer thickness (A, B, C, D) of LS2-FA samples after 1, 3, 7 and 28 day immersion in DMEM medium.

the start of precipitation. It means that apatite formation was not too rapid, nor did excessive ion leaching occur. As indicated by Hench & Andersson [42], for a medium-level bioactivity rate, it may be ideal for osteoblast survival, proliferation, nodule and ultimately bone formation. This claim was also confirmed by Gough *et al.* [43]. Together with his co-workers he reported that the presence of serum proteins can slow or inhibit Ca-P layer formation.

Overall comparison of apatite layer in SBF and DMEM

The formation of an apatite layer on the surfaces of glass-ceramic samples after various times of assay in SBF and DMEM media, as have been proved by SEM-EPMA analysis, is shown in Figure 9. The thicknesses of the surface layer were determined from two samples of the same composition, and the values plotted in the figure are the average values.

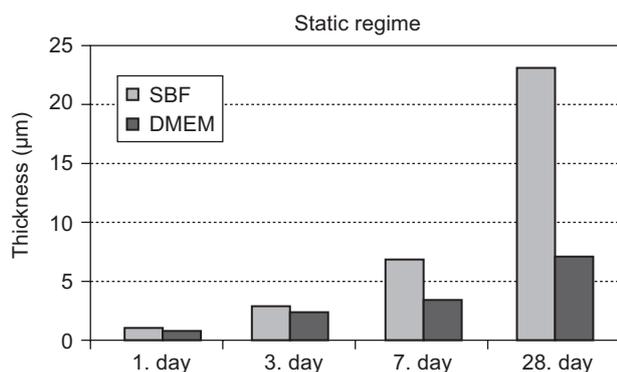


Figure 9. Comparison of thickness layers formed on the surface of LS2-FA samples after immersion in SBF and DMEM media.

The graphs clearly show that the growth of the apatite layer is much higher for all samples immersed in SBF than in DMEM medium. For the samples immersed in DMEM, the onset of apatite formation was slightly delayed in comparison to SBF. This is due to the presence of proteins in the medium. Generally we can observe a gradual increasing of thickness with time of immersion.

CONCLUSION

In this study, we have prepared and characterized LS2-FA glass-ceramic samples belonging to the $\text{Li}_2\text{O-SiO}_2\text{-CaO-P}_2\text{O}_5\text{-CaF}_2$ system in terms of *in vitro* bioactivity with immersion time in SBF and DMEM media. *In vitro* bioactivity was evaluated by monitoring changes in pH, silicon, calcium, phosphorous and lithium concentration in SBF and by analyzing newly

created layer on the surface of samples and its thickness confirmed by SEM and EPMA analyses. The outcomes of this research can be summarized as follows:

- The ICP and pH measurements for glass-ceramics samples with up to 28 days assay in the SBF medium clearly showed that Ca^{2+} and PO_4^{3-} have migrated to the surface through the SiO_2 rich layer forming a $\text{CaO-P}_2\text{O}_5$ rich film on top of the SiO_2 rich layer, followed by growth of an amorphous $\text{CaO-P}_2\text{O}_5$ rich film by incorporation of soluble calcium and phosphate from solution.
- The measured percent of solubility are steady and seems not to alter the overall solubility process of the silicate network and the glass-ceramic itself. This behavior initiates the processes for ionic exchange, silica gel layer and subsequent apatite layer formation.
- The reaction rate of layer formation in both media was different and was related to the immersion time.
- In SBF medium an apatite layer formed by gradual deposition of nano spherical small bullets that cover the whole surface during immersion period.
- Apatite formation in DMEM was similar to that formed in SBF, but in addition the surface was covered by globular aggregates with a non-regular morphology. For the samples immersed in DMEM, the onset of apatite formation was slightly delayed in comparison to SBF. The presence of proteins in DMEM may be the fundamental source that inhibits the apatite formation.
- The thickness of apatite-layer on the surface varies between 980 nm to 23.0 µm for LS2-FA glass-ceramic after immersion in SBF and between 680 nm to 7.00 µm for samples after immersion in DMEM.

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