

COMPARISON OF BIOACTIVITY *IN VITRO* OF GLASS AND GLASS CERAMIC MATERIALS DURING SOAKING IN SBF AND DMEM MEDIUM

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*This paper investigated the surface reactivity of two sets of glasses and glass ceramic materials belonging to the $\text{Li}_2\text{O}-\text{SiO}_2-\text{CaO}-\text{P}_2\text{O}_5-\text{CaF}_2$ system. The *in vitro* bioactivity of coatings was evaluated using simulated body fluid (SBF) and Dulbecco's Modified Eagle's Medium (DMEM) soaking test in static regime for up to 28 days at 36.5°C in microincubator. The surface structure changes were examined by scanning electron microscopy (SEM) and electron probe micro-analyzer (EPMA) methods. The functional groups of the silicate and phosphates were identified by infrared spectroscopy (IR). The crystal phases of the glasses and glass ceramics were identified by X-ray diffraction analysis (XRD). The results suggest the bioactivity behavior for all compositions of glasses as well as glass ceramic samples after 28 days in the SBF and DMEM medium. The surface characterization and *in vitro* tests revealed a few variations in the reactivity of the different glasses and glass ceramic samples in their pristine form. The best results show the samples of glass and glass ceramic samples with higher content of fluorapatite (FA). The use of the acellular culture medium DMEM resulted in a delay at the start of precipitation.*

INTRODUCTION

Bioactive glasses and glass ceramics represent a class of attractive materials for applied in medicine to repair and replace diseased or damaged bones or teeth [1]. In 1969 Hench *et al.* used the term “bioactive glasses” to describe this interfacial bond which developed between the implant and host tissue [2, 3]. At the same time, Hench developed the concept of using a silicate-based material with calcium and phosphate in proportions identical to natural bone as an implant material. It was found that after implantation in bone tissue, these materials resisted removal from the implant site and were, in effect, “bonded to bone” [4]. *In vivo*, this bonelike HA plays an essential role in the formation, growth and maintenance of the bone tissue-biomaterial interface, and can increase the bonding intensity [5, 6]. *In vitro*, this HA can enhance cell adhesion and stimulate cell proliferation [7].

The main advantage of bioactive glass is the induction of quick and direct interfacial bonding to the hard tissue due to biological equivalence of inorganic components of the mineralized tissue and the growing HA on the bioactive material surface [8]. Bioactive materials like silicate glasses and glass ceramics of specific compositions can directly bond to living bone

tissue depositing an intervening biologically active apatite layer when embedded in human body. Ca–Si based bioactive glasses are biodegradable and can form bone-like HA layer on its surface in simulated body environment [7]. When those materials are soaked into physiological solutions, silica gel layer with high surface area will be firstly formed on the materials surface by partial glass network dissolution and surface polycondensation, and it has been recognized that the silica gel layer plays an important role in the nucleation and growth of crystal hydroxycarbonate apatite (HCA) [9].

However, it should be stressed that bioactivity is not only a material property but also depends on the solution used for *in vitro* tests. Many efforts have been made by researchers, Hench [10] and later Davies [11], to understand the effect of solution type and material composition on HCA layer formation. *In vitro* studies are widely used for the study of bioactive implant materials because such tests allow prediction of the approximate behavior of such materials *in vivo* [12]. For example, Kokubo *et al.* have shown that a tris-buffer solution did not produce a HCA layer on bone-bonding apatite/wollastonite (A/W) glass ceramic. However, exposure of A/W glass ceramic to a simulated body fluid that contained ions in concentration similar to those of the human body produced a polycrystalline HCA layer [13].

Synthetic body fluids, prepared in accord with the chemical analysis of human body fluid, with ion concentrations and pH nearly equal to those of the inorganic constituents of human blood plasma, were first used by Kokubo *et al.* [14] in 1990, to prove the similarity between *in vitro* and *in vivo* behavior of certain glass ceramic compositions. In these studies, the glass ceramic samples were soaked in SBF solutions, and their surfaces were observed to be coated with the poorly crystallized calcium deficient and carbonate containing apatite, which was similar to bone apatite [15]. The fluids usually chosen to simulate plasma do not contain proteins. Addition of proteins to the fluid in contact with implant materials may affect mineralization through adsorption on materials and/or formation of complexes with dissolved ions, namely calcium, in physiological conditions. The biomaterial surface can be quickly coated with protein before other interactions occur, thus modifying the reactions with the environment [16]. The presence of proteins in blood is considered to be important in establishing the acceptance or rejection of an implant when placed *in vivo*. When an implant is placed into the body, proteins immediately become adsorbed on the surface of the material, which gives an indication of the clinical success of an implant in the body [17].

Kokubo *et al.* also claimed that the SBF method is useful for predicting the *in vivo* bone bioactivity of the material, not only qualitatively but also quantitatively [18]. In 2009, Bohner and Lemaître published a review paper entitled "Can bioactivity be tested *in vitro* with SBF solution?" which questioned whether there was currently enough scientific evidence to support the assumptions around the use of the SBF method. The paper concluded that although the use of SBF was valid the variability in the way the tests were carried out left room for improvement [19]. The findings collated by Bohner and Lemaître indicated that for the most significant mineral bone substitutes used *in vivo* (Bioglass, β -TCP, HA), bioactivity testing with SBF may lead not only to false positive but also to false negative results. The authors reported that serum and SBF are supersaturated towards apatite crystals and as such, the system is metastable and will thermodynamically stabilize by the formation of apatite crystals. Consequently, the validity of use of the SBF method to predict the *in vivo* bone bonding ability of a material may be open to question [20].

The poor mechanical strength of bioactive glasses is a major problem that limits their application as load-bearing implants. Approaches to achieve enhanced mechanical and biochemical properties include transformation of bioactive glasses into glass ceramic. In this technique, the glasses are subjected to thermal treatments which may affect the materials microstructures and hence their mechanical properties, but also their biological activity. In the present study different sets of glasses and glass ceramic samples belonging to the

$\text{Li}_2\text{O}-\text{SiO}_2-\text{CaO}-\text{P}_2\text{O}_5-\text{CaF}_2$ system have been prepared. The *in vitro* surface reactivity of the two sets of glasses and glass ceramic samples, with the lowest (0 % FA) and the highest (33.15 % FA corresponding to 14 wt.% P_2O_5) content of FA, have been investigated. Generally, SBF (which contains ions similar to blood plasma) is used as a medium for the development of biomimetic apatite. An attempt has been made for the first time to utilize conventional acellular fluid DMEM (which in addition to containing similar ionic concentrations as blood plasma also contains growth factors, proteins, hormones and vitamins common to blood) as a medium for the development of a bone-like apatite layer on the surface for the above mentioned samples of glass and glass ceramics.

EXPERIMENTAL

Materials synthesis

Glasses with different content of fluorapatite (FA), belonging to the system $\text{Li}_2\text{O}-\text{SiO}_2-\text{CaO}-\text{P}_2\text{O}_5-\text{CaF}_2$, were prepared by traditional melting technical a mixture of raw materials in a platinum crucible in a supercathal furnace at 1450°C (2 h, 10°C/min) with intermediate grinding and with a calcination step (5 h at 950°C). The mixture of raw materials contained: Li_2CO_3 (≥ 98 wt.%, Fluka, USA), ground quartz sand (SiO_2 , 99.6 wt.% SiO_2), dried CaF_2 (99.9 wt.%, Sigma-Aldrich, USA) and $\text{Ca}_3(\text{PO}_4)_2$ (96%, Fluka, USA) (Tab. 1). The ratio of CaF_2 and $\text{Ca}_3(\text{PO}_4)_2$ responses to the stoichiometric FA composition. Pure lithium disilicate (shorthand LS_2) glass without P_2O_5 and CaF_2 (i.e. without FA) content was prepared as a reference sample. 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, re-melted and poured into of copper moulds to form discs with precisely defined dimensions as listed above.

Table 1. Glass compositions (wt.%).

Components	FA content (wt.%)	
	0	33.15
SiO_2	61.93	44.76
Li_2CO_3	38.07	27.52
CaF_2	–	2.15
$\text{Ca}_3(\text{PO}_4)_2$	–	25.58

Representative samples of glass ceramics with the lowest and highest content of FA were prepared by annealing, or thermal treating of parent glasses under optimized regime in a muffle furnace at 600°C for 6 hours (heating rate 10°C/min) as reported in [21]

to characterize the crystallization course of different phases. Finally the glass and glass ceramic samples were cut into rectangles with dimensions 0.6 cm in length, 0.5 cm in width and 0.5 cm in thickness.

In vitro bioactivity

The assessment of *in vitro* bioactivity was carried out by soaking glass and glass ceramic samples in two media, simulated body fluid (SBF) and Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, Germany) maintained at 36.5° in incubation apparatus (Binder BD 115). Soaking period was 28 days under static regime. Specifically, the samples were soaked in 25 ml of SBF and DMEM medium in sterilize polyethylene bottles. DMEM matches more closely the biological

conditions due in particular to the presence of amino acids. DMEM is also the culture medium that will be used for future cell interactions studies.

Simulated body fluid is an acellular, aqueous solution with an ionic composition that closely resembles that of human plasma and buffered to physiological pH 7.25-7.4. Each small undesired variance in the preparation steps and the storage temperatures, may drastically affect the phase purity and high temperature stability of the produced HA on the surface, as well as the kinetics of the precipitation processes. SBF was prepared by dissolving the components NaCl, NaHCO₃, KCl, K₂HPO₄·3H₂O, MgCl₂·6H₂O, CaCl₂·6H₂O, Na₂SO₄ per litre of ultrapure water in a beaker according to the method developed by Kokubo *et al.* [18]. It was buffered at pH 7.25 with tris(hydroxymethyl)-ami-

Table 2. Compositions of the human blood plasma, acellular culture medium DMEM and SBF [22].

Ion concentrations (mM/l)	Na ⁺	K ⁺	Ca ²⁺	Mg ²⁺	HCO ₃ ⁻	Cl ⁻	HPO ₄ ²⁻	SO ₄ ²⁻
Blood plasma	142.0	3.6-5.5	2.1-2.6	1.0	27.0	95.0-107.0	0.65-1.45	1.0
DMEM	154.56	5.37	1.82	0.8	44.0	120.5	1.0	0.8
SBF	141.8	5.0	2.5	1.5	4.2	148.0	1.0	0.5
	Blood plasma		DMEM		SBF			
pH	7.25 - 7.4		7.3		7.4			
Buffer	No		No		Tris(hydroxymethyl) aminomethane + HCl at 36.5 °C			
Compounds (mg/l)	Alanine (20.5-40.1) Arginine (2.3-11.2) Asparagine (6.0-17.2) Aspartic acid (0-0.8) Cystine (7.2-15.6) Glutamic acid (2.7-14.4) Glutamine (57.0-95.0) Glycine (12.8-24.8) Hystidine (4.0-18.6) Isoleucine (5.5-13.1) Leucine (8.7-22.3) Lysine (21.9-32.2) Methionine (2.4-4.5) Phenylalanine (6.7-11.2) Proline (12.6-41.5) Threonine (11.0-28.6) Tyrosine (8.2-13.4) Valine (17.6-36.3) Urea (132.1-438.4) Uric acid (<70.6) Creatine (1.6-4.0) Creatinine (<14.0) Glucose (549.5-1153.1) Cholesterol (1082.6-2010.6) Triglyceride (<1368.6) Serine (5.9-14.7) and others		L-Arginine HCl (84) L-Cystine (48) L-Alanyl-L-Glutamine (862) Glycine (30) L-Histidine HCl H2O (42) L-Isoleucine (105) L-Leucine (105) L-Lysine HCl (146) L-Methionine (30) L-Phenylalanine (66) L-Serine (42) L-Threonine (95) L-Tryptophane (16) L-Tyrosine (72) L-Valine (94) D-Calcium pantothenate (4) Choline Chloride (4) Folic Acid (4) i-Inositol (7.2) Nicotinamid (4) Pyridoxine HCl (4) Riboflavine (0.4) Thiamine HCl (4) D-Glucose (1000) Phenol red (15) Sodium Pyruvate (110)		No			

nomethane ((HOCH₂)₃CNH₂) and hydrochloric acid (HCl). SBF, DMEM and plasma are saturated with respect to hydroxyapatite. The composition of SBF has been presented in Table 2 along with DMEM and human blood plasma for comparison purposes.

After exposure in SBF and DMEM medium, the glass and glass ceramic samples were taken out from the incubator and rinsed gently with distilled water and pure ethanol. Then the samples were dried at ambient temperature inside the desiccator for further analysis.

Experimental methods

The glass and glass ceramic samples before and after immersion in SBF and DMEM medium were mounted on aluminium stubs with double sided carbon tape, ion sputtered with a thin layer of gold and examined for their size and microstructure (pore size, shape and

interconnectivity) in scanning electron microscopy (SEM - TESLA BS 300 with digital unit TESCAN). The electron probe micro-analyzer analysis (EPMA JEOL JXA-840A, EDS parameters - 15KV, Takeoff Angle 40.0°) was used to analyze the surface layer formed on the samples before and during exposure in SBF and DMEM medium. Samples were carbon coated before analysis. The IR spectrum of synthetic samples was recorded using a Nicolet 6700 FT-IR Spectrometer, using the KBr pellet technique. Samples were mixed with KBr powder in a weight ratio of 4:250 mg and pressed into pellets and analyzed at a resolution of four wavenumbers, operating from 4000 to 400 cm⁻¹. The nature and morphology of the crystalline phases in the samples were investigated through X-ray diffraction (XRD - D710, Siemens, using CoK radiation with a wavelength of $\lambda = 1.788$ nm, operating at 40 kV and 30 mA Germany). The glass and glass ceramic samples were ground into fine powder before testing by XRD.

RESULTS AND DISCUSSION

Phase analysis (XRD)

Glass and glass ceramic samples (without and with 33.15% FA) before immersion in biological fluids were analyzed by X-ray diffraction (Figure 1).

The XRD patterns for glass samples (Figure 1a) show an amorphous character of samples. The glass heat-treated samples at 600°C (Figure 1b) was successfully crystallized into LS₂ glass ceramic with its characteristic XRD peaks. The one crystal phase in this glass ceramic samples was identified as LS₂ crystal phase (JCPDS 17-0447 with $d = 3.67(100), 3.21(80), 3.50(60)$ and $3.60(2)$ Å). The crystallization is well developed at this temperature. Fluorapatite remains in amorphous state. Kuzielová *et al.* [21] demonstrated that LS₂ crystallizes firstly at lower temperatures, while FA crystals are formed at higher temperatures and at the same time that P₂O₅ at lower concentration acts as nuclear agent for LS₂ crystallization and promote it via surface mechanism.

In vitro bioactivity

Morphology and composition (SEM, EPMA) of glass samples before and after 28 days immersion in SBF and DMEM medium

Biomimetic coatings precipitated on the glass sample (without and with 33.15% FA) surface were analyzed by SEM. A representative micrograph accompanied by the EPMA spectra are shown in the right corner of Fig. 2.

Before immersion into biological fluids, as it can be seen in Figure 2a-d in both glass samples, the surface is very uniform and homogeneous. As the results of EPMA analysis shows, surface of glass without FA

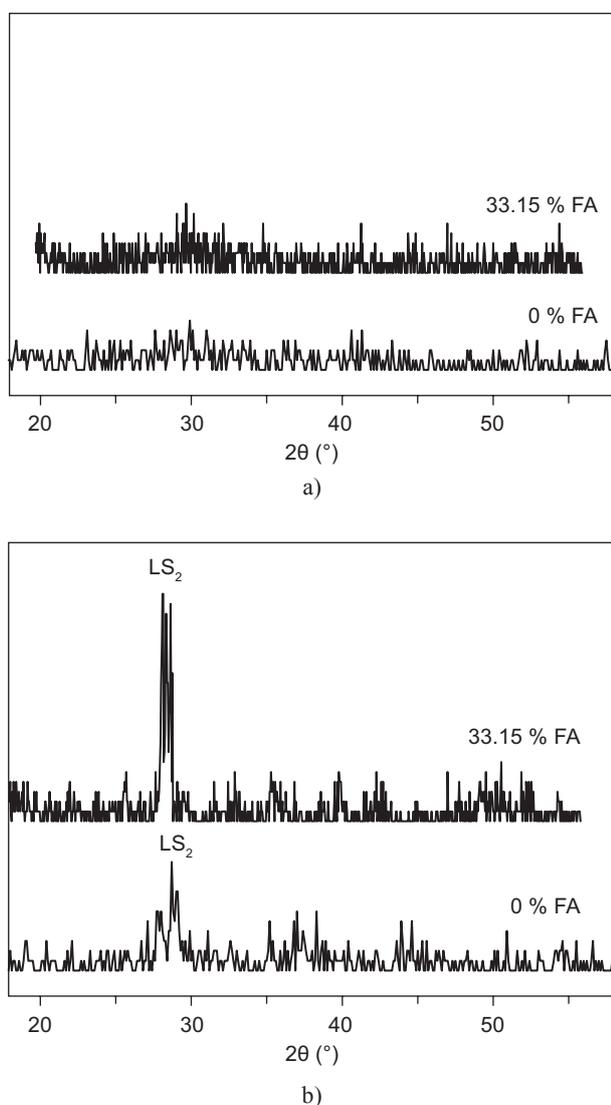


Figure 1. a) XRD diffraction patterns of glass samples with different FA content (wt.%); b) XRD diffraction patterns of glass ceramic samples heated for 6 h at 600°C.

addition and before immersion in biological fluids is characterized by a dominant presence of Si. The glass sample with 33.15 % FA before immersion in biological fluids is characterized also by a dominant presence of Si together with Ca and P originating from mixture of CaF_2 and $\text{Ca}_3(\text{PO}_4)_2$ in the batch with stoichiometric ratio corresponding to FA.

In comparison with the smooth-faced surfaces of primary glasses, the surfaces of glasses after *in vitro* test in SBF and DMEM medium have changed. The surface morphology and EPMA analysis on the glass without FA after 28 days of immersion in SBF and DMEM medium without refreshing the solution, the layer formed on the surfaces is characterized by a dominant presence of Si and a small presence of Ca and P (Figure 2b,c). Samples without FA content show surface partially covered with dispersed regions of new phases. The surface microstructure changes with FA, expressed as P_2O_5 in samples. Indeed, authors [23, 24] have demonstrated that the presence of FA supports the crystallization via surface mechanism. However the increased amounts of Ca and P from EPMA analysis may indicate the onset of the formation of an amorphous $\text{CaO-P}_2\text{O}_5$ rich layer (Figure 2e,f). One can see, that the HA structure was more pronounced in sample with 33.15% FA and after 28 days of immersion in SBF. Whereas in DMEM medium, small spherical forms characteristics for HA were formed on

the surface of samples. The average Ca/P atomic ratio in SBF was calculated ($\text{Ca/P} = 1.80$). The Ca/P atomic ratio rose to 1.96 with increasing concentrations of HCO_3^- in DMEM medium (Table 3). Besides these major elements, the presence of small amount of Na, Cl and Mg was detected. The above components were derived from SBF and DMEM medium.

Table 3. EPMA analysis of glass samples before and after 28 days of immersion in SBF and DMEM medium.

Element	Measured content (atomic %)		
	before immersion	SBF immersion	DMEM immersion
<i>glass 0 % FA</i>			
Si	33.20	22.98	32.56
Ca	–	7.70	0.34
P	–	3.75	0.47
Ca/P	–	2.05	0.72
<i>glass 33.15 % FA</i>			
Si	22.65	–	1.99
Ca	9.19	24.36	20.99
P	3.72	13.55	10.69
Ca/P	2.47	1.80	1.96

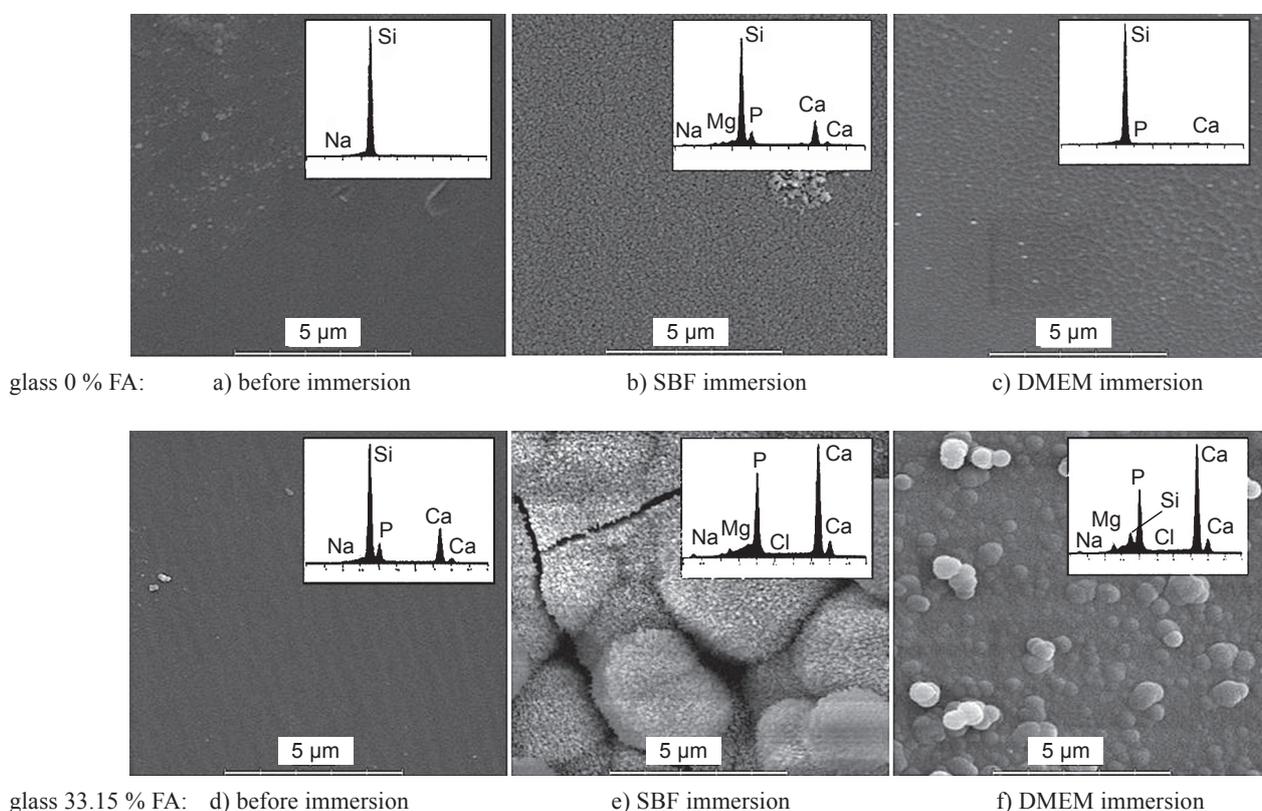


Figure 2. SEM - EPMA surface analysis of glass samples before and after 28 days of immersion in SBF and DMEM medium.

*Morphology and composition (SEM, EPMA)
of glass ceramic samples before and after
28 days immersion in SBF and DMEM medium*

Figure 3 reports the results of SEM-EPMA analysis performed on glass ceramic samples (without and with 33.15% FA). Before immersion into biological fluids (Figure 3a-d) the microstructures are fine grained, compact and consist of one type of crystal. It can be observed a dominant presence of Si on the surface of both samples. The chemical composition logically is related to the reported in Table 1. In sample with 33.15% FA one can note the dominant presence of Si on the surface. Next to Si, Ca and P presence is detected. This is due to the presence of FA crystallized on the surface of glass ceramics.

The surface behavior of glass ceramic sample without FA after 28 days soaking in SBF and DMEM medium (Figure 3b,c) was similar to that previously mentioned for glass sample without FA. The surface was partially covered by the new phases. Also by the EPMA analysis, small amount of Na, Cl and Mg was detected. The above components were derived from SBF and DMEM medium.

The morphology of glass ceramic surface changed distinctly with respect to that of initial glass ceramic

surface. In the case of the samples soaked in SBF and DMEM medium, without refreshing the solution, one can observe the structural changes which took place on the surface and different globular agglomerates on the surface (Figure 3e,f). The entire surface of the samples after immersion is covered by small spherical particles which form a continuous layer of HA. With increasing content of FA (33.15% FA), the surface morphology and

Table 4. EPMA analysis of glass ceramics samples before and after 28 days of immersion in SBF and DMEM medium.

<i>glass ceramics</i> 0 % FA	Measured content (atomic %)		
	before immersion	SBF immersion	DMEM immersion
Element			
Si	33.33	31.93	32.82
Ca	–	0.46	–
P	–	0.61	0.38
Ca/P	–	0.75	–

<i>glass ceramics</i> 33.15 % FA	Measured content (atomic %)		
	before immersion	SBF immersion	DMEM immersion
Element			
Si	22.62	–	4.96
Ca	8.17	25.14	24.88
P	4.26	13.15	11.09
Ca/P	1.92	1.91	2.24

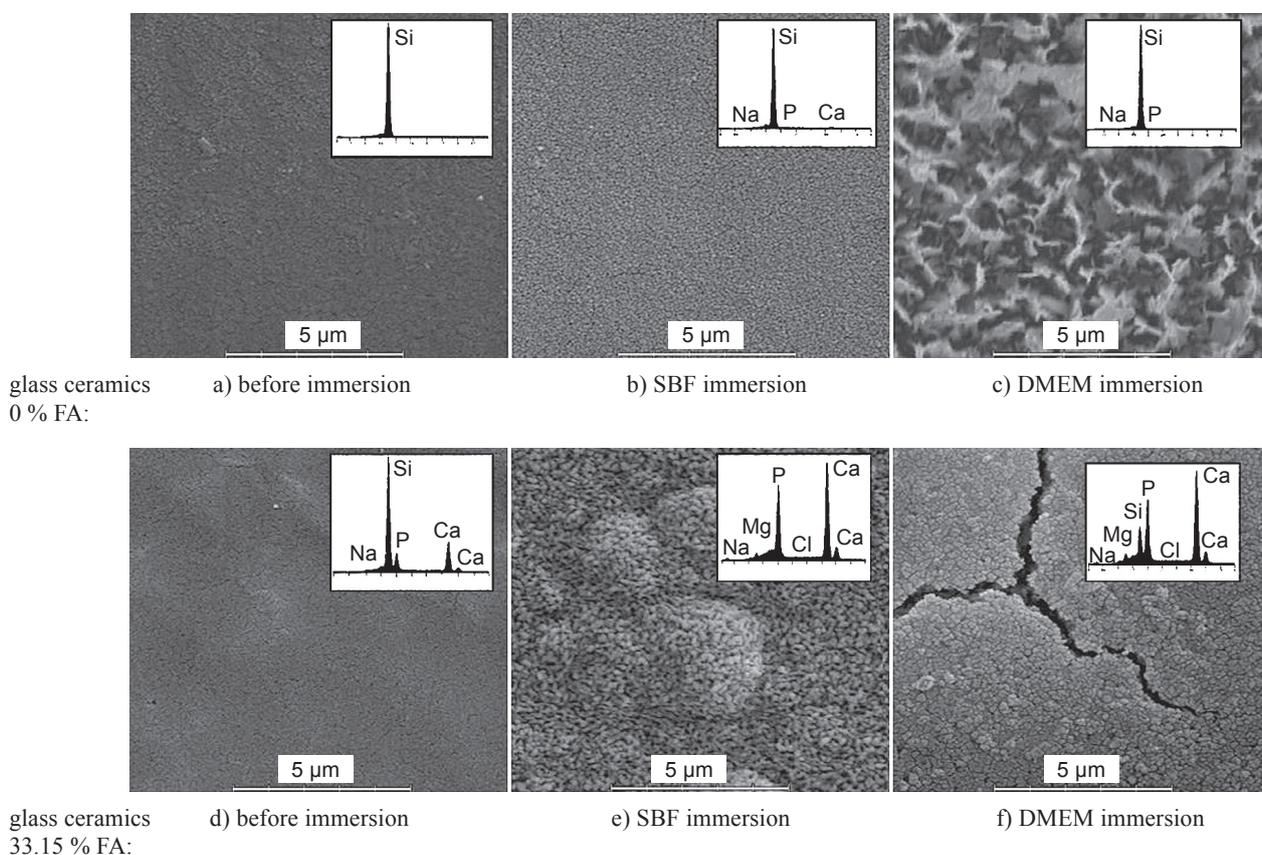


Figure 3. SEM - EPMA surface analysis of glass ceramics samples before and after 28 days of immersion in SBF and DMEM medium.

EPMA analysis after 28 days of immersion in SBF and DMEM medium showed high reactivity by forming a calcium phosphate-rich layer on their surfaces. EPMA revealed pronounced CA and P peaks as well as other peaks corresponding to elements found in parent glass. The average Ca/P atomic ratio in SBF was calculated (Ca/P= 1.91). The Ca/P atomic ratio rose to 2.24 (Table 4). Peitl *et al.* [13] have found that glass ceramics with crystalline phases are less reactive than related glasses. In some case, the crystallization can even turn a bioactive glass into inert biomaterial.

Heat treatment of this sample makes it possible to prepare glass ceramics with improved hardness and probably also mechanical strength, but bioactive properties are not similar to those of the initial glass. Though the content of FA is sufficient to induce a relatively high rate of apatite formation, the surface transformation related to heat treatment diminishes the bioactivity of glass ceramics. With precise heat treatment the optical properties of the resultant glass ceramics can be also controlled and adjusted to the desired requirements.

Analysis of functional groups (IR) in glass and glass ceramic samples without FA before and after 28 days immersion in SBF and DMEM medium

IR spectra of the glasses and glass ceramic samples without FA before and after immersion in biological fluids are shown in Fig. 4. The peak assignments of the various vibrational modes observed in these materials are listed in Table 3.

The main characteristics of the spectrum not soaked bioglass samples are attributed to the amorphous silica glass, e.g. the strong band at 1036 cm⁻¹ in the spectra is known to be caused by the highest frequency component

of the asymmetric stretching mode of the Si–O–Si [25]. The band at 947 cm⁻¹ is known to be caused by the non-bridging oxygen stretching mode of the Si–O⁻ [26]. The presence of non-bridging oxygen stretching mode is the main requirements for initiation of bioactive process. Their concentration controls the rate of silicate matrices leaching (degradation - decomposition) leading thus to the formation of silan group at the surface of glass [27]. The band at 777 cm⁻¹ is due to the symmetric stretching of Si–O–Si bond, while the band at 480 cm⁻¹ can be assigned to the bending vibration of the O–Si–O bend [28]. Peaks for OH⁻ groups and adsorbed water at the surface were also noted [27]. In comparison with the not soaked glass ceramic samples, crystallized conventionally at 600°C for 6 hours, the spectra with many expressive intensity of peaks has been identified as LS₂ crystal phase when compared to the literature [29]. The most noticeable changes in the IR spectra relative to the amorphous glass are seen between 700 cm⁻¹ and 400 cm⁻¹. The assignment of the IR bands is given in Table 5.

Table 5. IR bands for LS₂ glass ceramics [29].

IR bands (cm ⁻¹)	Assignment of the IR bands
1213	(Si–O–Si) asymmetric stretching
1108	(Si–O–Si) asymmetric stretching
1028	(Si–O–Si) asymmetric stretching
939	(Si–O–Si) asymmetric stretching
825	(Si–O–Si) symmetric stretching
760	(Si–O–Si) symmetric stretching
637	(Si–O–Si) symmetric stretching
553	(Si–O–Si) symmetric stretching
548	(O–Si–O) bending mode
469	(O–Si–O) bending mode
409	(O–Si–O) bending mode

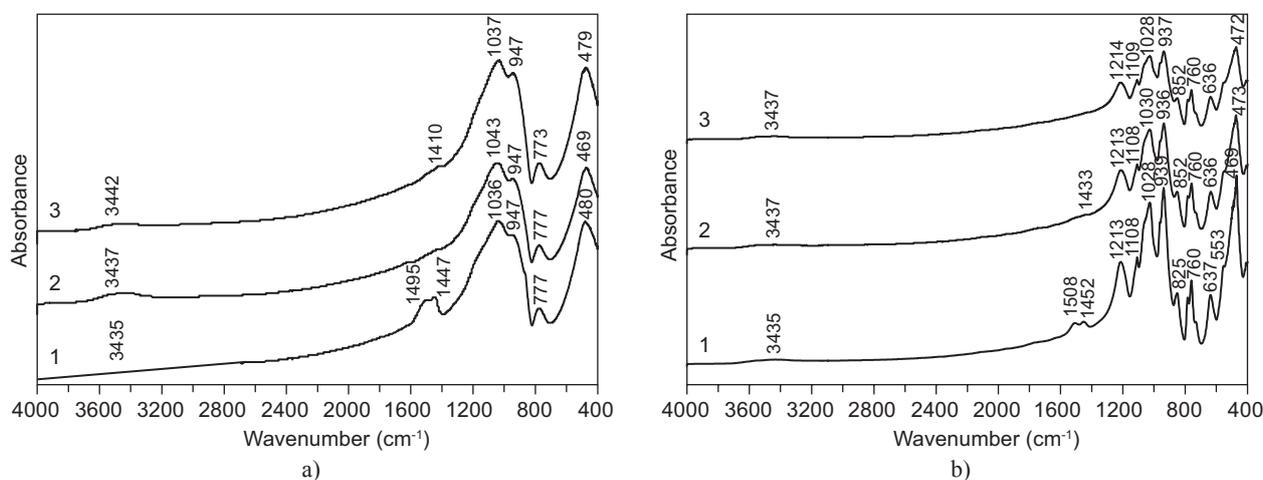


Figure 4. IR spectrum of glass (a) and glass ceramic (b) without FA before immersion (1), after 28 days in SBF (2) and after 28 days in DMEM medium (3).

The IR characteristic for both biological measurements yielded similar spectrum of glass and glass ceramic samples although with different band intensities. The absorption spectra of the soaked glass and glass ceramics samples after 28 days in SBF or DMEM medium reveal new bands that can be assigned to CaO–P₂O₅ rich layer on the bioglass samples surface. Actually, the strong band at ~1043 cm⁻¹, ~1030 cm⁻¹ for SBF and ~1037 cm⁻¹, ~1028 cm⁻¹ for DMEM is assigned to P–O stretching vibration and one at 600–550 cm⁻¹ assigned to P–O bending vibration for the amorphous phase layer. Moreover, carbonate absorption bands at about ~1417 cm⁻¹, ~1433 cm⁻¹ for SBF and ~1410 cm⁻¹ for DMEM are also detected [30]. IR analysis of both samples showed the formation of carbonate bands for the samples soaked in SBF. However the evolution of significant carbonate bands was not seen or is slower for samples soaked DMEM. One can see, that a silica-rich layer is present together with a thin carbonate-containing HA layer as demonstrated by the results of SEM and EPMA analysis.

Analysis of functional groups (IR) in glass and glass ceramic samples with 33.15% FA before and after 28 days immersion in SBF and DMEM medium

The IR characteristic of glass and glass ceramic samples with 33.15% FA before and after immersion in SBF and DMEM medium were performed to study the HA layer formation on their surfaces (Figure 5).

For the glasses and glass ceramics containing phosphate before immersion, a broad absorption band at 3450 cm⁻¹ and the bending mode at 1650 cm⁻¹ related to existence of H₂O impurity, and it is because of KBr humidity. A broad phosphate band derived from the P–O asymmetric stretching mode (ν_3) of the (PO₄)³⁻ group

was found in the region from 1200 to 960 cm⁻¹ indicating a deviation of the phosphate ions from their ideal tetrahedral structure. The band identified at 943 cm⁻¹ corresponds with symmetric valence vibration (ν_1) of phosphate group [31]. The spectra peaks are more shifted comparing with those belonging to pure LS₂ glass. This is due to partial crystallization of glass during cooling. Our previous work [21] has revealed that the presence of FA supports the crystallization of glass via surface mechanism. The absorption bands found at 561 cm⁻¹ and 631 cm⁻¹ can be assigned to ν_4 of the (PO₄)³⁻ vibration of phosphate compounds, likewise, bands at 474 cm⁻¹ and at 453 cm⁻¹ are similar to ν_2 of the (PO₄)³⁻ vibration [32]. Apart from the presence of orthophosphate, one can observe some bands characterizing the presence of triply and doubly degenerated bending modes of phosphates. The bands at 761 cm⁻¹ and 784 cm⁻¹ that can be assigned to ν_s (P-O-P) demonstrate the presence of aforementioned compounds [33]. The band at 936 cm⁻¹ is assigned to ν_1 of the (PO₄)³⁻ vibration [13]. The absorption band at 1209 cm⁻¹ can be assigned to ν_{as} or ν_s vibration of PO₃ or PO₂ groups from diphosphate and more condensed phosphate compounds. The band at 868 cm⁻¹ in spectra characterizes the ν_2 (CO₃)²⁻ vibration [27].

After 28 days immersion in SBF or DMEM medium, some splitted bands appear on the IR spectra. The band near 1026 cm⁻¹ ν_s (PO₂) and band at 961 cm⁻¹ or at ~936 cm⁻¹ ν_3 (PO₄)³⁻ [33].

The last two bands that are assigned to ν_4 (PO₄)³⁻ vibration (two bands at ~629 cm⁻¹ and ~561 cm⁻¹) and ν_2 (PO₄)³⁻ (~475 cm⁻¹) [32] indicate the formation of well crystallized HA carbonated. Incorporation of carbonate ions from solution SBF or DMEM to apatite structure is demonstrated by the presence of ν_3 (CO₃)²⁻ and ν_2 (CO₃)²⁻ that appear at 1433 cm⁻¹ and 852 cm⁻¹ (data not shown) [27].

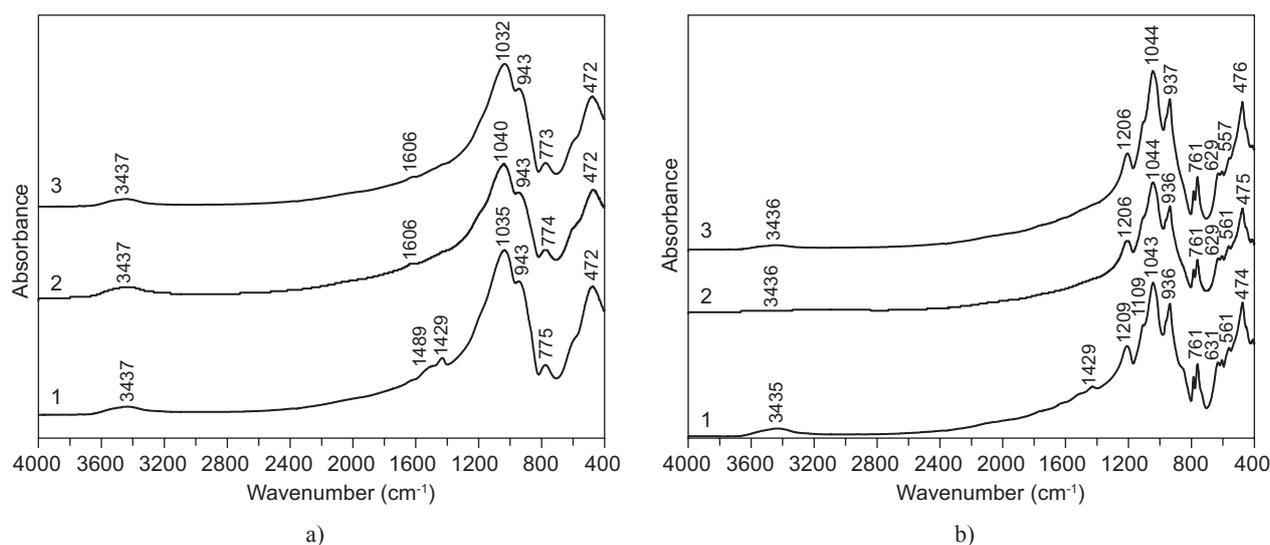


Figure 5. IR spectrum of glass (A) and glass ceramics (B) with 33.15% FA before immersion (1), after 28 days immersion in SBF (2) and after 28 days immersion in DMEM medium (3).

In addition to aforementioned bands characterizing the presence of HCA, some ν_{as} (P–O–P) bands at $\sim 784\text{ cm}^{-1}$ and at $\sim 761\text{ cm}^{-1}$ corresponding to ν_s (P–O–P) were identified [33] after 28 days immersion in SBF and DMEM. The development of HCA layer caused the weakening and finally the disappearance of the bands attributed to Si–O–Si vibration from bioactive glass. The large absorption band in 3436 cm^{-1} in regions characterizes the presence of δ (H_2O) [34].

CONCLUSION

In this paper, we have prepared and characterized two sets of glasses and glass ceramics belonging to the $\text{Li}_2\text{O–SiO}_2\text{–CaO–P}_2\text{O}_5\text{–CaF}_2$ in terms of composition, *in vitro* bioactivity and phase analysis.

After 28 days immersion in biological fluids the SEM and EPMA measurements indicate the formation of micro-crystalline HA phase. These results are at the origin of crystallization of the amorphous $\text{CaO–P}_2\text{O}_5$ film by incorporation of OH^- and CO_3^{2-} anions from solution to form hydroxyl carbonate apatite layer. Hydroxyapatite formation in DMEM was similar to that formed in SBF, although the process was slightly slower in this culture medium. The role of proteins in promoting or inhibiting (or even both) the formation of HA and the processes of biomineralization are not clear, although it has been reported that the presence of serum proteins can slow or inhibit HA formation [35]. The results suggest that HA formation should not be too rapid, nor should excessive ion leaching occur, i.e., a medium-level bioactivity rate may be ideal for osteoblast survival, proliferation, nodule and ultimately bone formation.

The most noticeable changes in the IR spectra in comparison glasses and glass ceramics samples relative to the amorphous glass and crystallized phases are seen between 700 cm^{-1} and 400 cm^{-1} . Comparing the IR spectra of glass and glass ceramics before and after immersion in biological fluid demonstrated the presence of carbonate groups, indicating the formation of a carbonated apatite at the surface. The phosphate peaks became more intense and sharp with the immersion time, indicating the growth of crystalline apatite *in vitro*. These results suggest that the apatite formed on the surface of specimens in SBF was carbonated apatite, which is similar in composition and structure to bone apatite and was also found on bioactive glasses.

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