STATIC AND DYNAMIC IN VITRO TEST OF BIOACTIVITY OF GLASS CERAMICS

GABRIELA LUTIŠANOVÁ, EVA KUZIELOVÁ, 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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The bioactivity of glass ceramics from $Li_2O–SiO_2–CaO–P_2O_5–CaF_2$ system, with different amount of fluorapatite expressed as P_2O_5 content, has been tested in vitro under static and dynamic regime. The paper reports the results of bioactivity test of glass ceramics in static and dynamic regime. XRD, SEM and EPMA analysis were used to characterise the sample as well as to detect the presence of new phase onto the surface of glass ceramics. The bioactivity, as demonstrated by the formation of new apatite layer, depends on P_2O_5 content and testing regime. In static regime, one can observe a fine microstructure of hydroxyapatite layer on the surface on glass ceramics samples. In dynamic regime, the formation rate of this layer seems to be retarded in comparison with that of static regime.

INTRODUCTION

The term of bioactivity of implant used in medicine was defined as following: "A bioactive material is one that elicits a specific biological response at the interface of the material which results in the formation of a bond between the tissues and the material" [1]. It is intermediate between resorbable and bioinert biomaterials. However, the time dependence of bonding, the strength of bond, the mechanism of bonding and the thickness of the bonding zone differ for the various biomaterials [2].

Many kinds of biomaterials based on glass ceramics have been developed and some of them are now applied to repair and replace diseased or damaged bones or tissues [3-6]. The advantage of glass ceramics is their good mechanical and bioactivity properties; their stability in biological environment and their facility do be synthesized and tailored from glass melt. Indeed, glass ceramics are obtained by controlled crystallization process of glass at suitable temperature. First the glass is obtained by cooling a glass melt that is easy for shaping to any form of materials. The shaped glass is then transformed into glass ceramics with required mechanical and optical properties [7, 8].

The authors [4, 9, 10] have developed a large range of glasses and glass ceramics in $Li_2O–SiO_2$ system with potential applications in medicine. Because of high strength, opalescence, thermal stability and chemical resistance, the glass ceramics of this type are applied as dental bridges, crowns or veneers. To improve the bioactivity of glass and glass ceramics in $Li_2O–SiO_2$ system, CaO, P_2O_5 and CaF_2 were added in different amount but in such proportion to form fluorapatite. The glass samples with defined dimensions were annealed at different temperatures to obtain glass ceramics. It was found that adding fluorapatite enhances the bioactivity of glass ceramics, but in the contrary, the annealing temperatures decrease it. For potential applications in medicine, these samples were submitted to the cytoxicity, genotoxicity and mutagenity study in cultural medium of living cellules [11].

The bioactivity *in vitro* test as proposed by Kokubo [12] is realized in a calculated volume of aqueous solution with pH = 7.4 and with similar chemical composition to inorganic salts as human blood. The system is kept at constant temperature of 36.5°C ± 1°C in the incubation apparatus during whole experimental period. In this *in vitro* test, biodegradation of samples or biomineralisation of apatite layer run under static regime.

The bioactivity characteristics of biomaterials are determined by observing (Scanning Electron Microscopy - SEM or Transmission Electron Microscopy - TEM)

and analysing (XRD, FTIR or EPMA) their surface after a certain period of immersion in simulated body fluid (SBF). Biomaterials used in implantological applications have to demonstrate besides the appropriate mechanical properties their bioactivity, hence the ability to promote bone tissue growth. Their bioactivity is attributed to the formation on their surfaces of a hydroxycarbonated apatite (HCA) layer similar to a large extent to the mineral part of bone. The rate of tissue bonding appears to depend on the rate of HCA formation, which follows a sequence of reactions between the implanted material and the surrounding tissues and physiological fluids [13]. The mechanism of the layer nucleation and growth proposed by Kokubo consists in the interchange between Ca^{2+} ions of the glass and the H_3O^+ of the solution. This gives rise to the formation of Si–OH functional groups on the glass surface inducing thus apatite nucleation. The nuclei thus formed grow at the expense of the ions in the solution saturated with respect to the apatite [10]. For instance, Li et al. [6] showed that a bioactive glass can be transformed into an inert glass ceramic. They found a HCA layer formation using *in vitro* tests only if the glass ceramics contained a high proportion (over 90 %) of the residual glassy phase. *In vitro* tests are intended for use in screening bone bioactive materials before animal testing. The number of animals used and the duration of experiments can be significantly reduced by using these methods, which can assist in the efficient development of new types of bioactive materials [1].

Unfortunately, the *in vitro* test proposed by Kokubo is realised in static condition that is far from the biological realities in human body. The concentration of ions in SBF decreases with time and thus apatite formation can be stopped. These facts are illustrated by the results of investigations realized by [14] on the influence of SBF composition upon the formation of carbonated hydroxyapatite on the glass surface. The ion concentrations and pH of aqueous solution varied with testing period. It results from this study that the kinetics and mechanism of hydroxyapatite formation are influenced, not only by immersion time, but also by the variation of ion concentrations and pH of SBF in static regime. Rámila et al. and recently Zhang et al [15, 16] have proposed a dynamic regime of *in vitro* test in which SBF is continuously renewed and circulated around the samples at determined speed. The system enables to keep constant the concentration of SBF in container with sample, as it is linked to a reservoir with solution of SBF.

Authors [17] have explored the same method to evaluate the bioactivity of bioglass in $CaO\text{-}SiO_2\text{-}P_2O_5$ system. The main advantages of the new system are that the pH values and the concentration of Ca^{2+} ions are stable in container with sample, while they change drastically in static regime. In static regime, the pH values and Ca^{2+} ion concentration increase with immersion time to reach maximum before to decrease. In a specific case, the variation of pH values is influenced by the content of HCO_3^{-1} as reported by [14]. Also, the microstructure of apatite layer and the degree of crystallinity have differed as demonstrated the results of XRD and SEM. The construction of this system does not approach enough the biological one, as the reservoir is placed outside the incubation apparatus with different temperature. To avoid the influence of temperature of SBF in reservoir upon the bioactivity of sample, we propose in this work to place the reservoir containing SBF inside the incubation apparatus keeping thus it at same temperature than that of SBF in container with samples (Figure 1). The samples are exposed to continual circulating SBF during test period.

EXPERIMENTAL

Preparation of glass ceramics

The samples of glass ceramics with different content of P_2O_5 were obtained by partial devitrification of parent glass. The glass samples were prepared by traditional melting process. The compositions of these glasses are given in Table 1. The ratio of CaF_2 and $Ca_3(PO_4)_2$ responses to the stechiometric fluorapatite composition. Pure lithium disilicate glass without P_2O_5 and CaF_2 content was prepared as a reference sample. The reactants were weighed and mixed in the proportions reported in Table 1. Then, the resulted suspensions were stirred by using the magnetic blender at current heating for 1 h. Suspension mixtures were dried under IR lamp and then in the oven at 105°C. The as prepared powdered samples were twice melted in a covered platinum crucible in a supercanthal furnace at 1450°C (2 h, 10°C/min) with intermediate grinding. Glass melts were poured onto anticorrosive 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. The glass samples were cut into rectangles of the dimensions 0.5 cm × 0.5 cm × 0.5 cm.

Samples of glass ceramics with different P_2O_5 content were prepared by thermal treatment of parent glasses under optimised regime in a muffle furnace at 550°C and 750°C for 6 hours (heating rate 10°C/min) as reported in [4] to characterize the crystallization course of different phases.

Table 1. The composition of mixtures designated for preparation of glasses (wt.%).

	P_2O_5 content (wt.%)				
Components	0	5	10	12	14
SiO_2 - Tosil	84.00	80.54	76.33	74.46	72.87
Li_2O	6.47	6.20	5.87	5.73	5.46
CaF_2	-	0.32	0.71	0.84	1.05
$Ca_3(PO_4)_2$	-	3.82	8.44	10.54	12.57

Preparation of simulated body fluid

In vitro assays of bioactivity under static as well as under dynamic regimes were performed by soaking the glass ceramic samples in simulated body fluid (SBF), an acellular aqueous solution with inorganic ion composition almost equal to human blood plasma (Table 2) [18].

SBF was prepared by dissolving the components 8.035 g NaCl, 0.355 g $NaHCO_3$, 0.225 g KCl, 0.231 g $K_2HPO_4 \cdot 3H_2O$, 0.311 g $MgCl_2 \cdot 6H_2O$, 0.292 g $CaCl_2 \cdot 6H_2O$, 0.072 g Na_2SO_4, 6.118 g $(HOCH_2)_3CNH_2$ per litre of ultra pure water in a beaker. The pH was adjusted using 1 M hydrochloric acid according to the method proposed by Kokubo et al. [12]. Sodium azide was added to inhibit bacterial growth. SBF was kept at 36.5°C in incubation apparatus.

The samples were poured in the plastic containers in a volume of model solution corresponding to $S_a/V_S \cong 10$ cm^{-1} [4, 14] and heated up to the temperature of 36.5°C ± 1°C.

The samples were immersed separately in the solutions of SBF under static (Figure 1a) and dynamic regimes (Figure 1b) and stored with constant volume in the incubation apparatus (Binder BD 115) for four weeks at the temperature 36.5°C ± 1°C. After exposure, the glass ceramic samples were taken out and washed gently with distilled water and ethanol. Then the samples were dried inside the desiccator for further analysis.

The dynamic regime, as illustrated in Figure 1b, is composed of pumping tank (3) containing SBF solution. SBF is continuously pumped by peristaltic pump (2) through silicone tube from pumping tank to reservoir (7), from which SBF solution fills container (5) through closing valves (6) at speed of 0.033 ml/min. The whole construction is located in incubation apparatus (4) at constant temperature of 36.5°C.

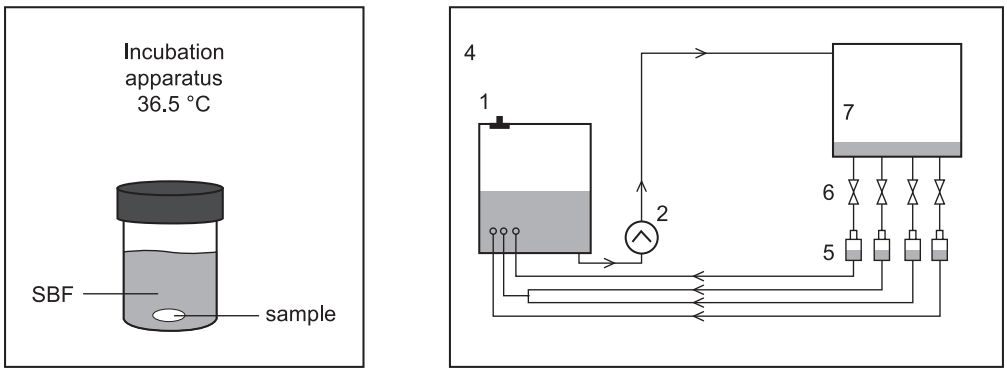
Table 2. Ionic concentrations in SBF and human blood plasma.

	Ion concentrations (mmol/L)	
Ion	Blood plasma	SBF
Na^+	142.0	142.0
K^+	3.6-5.5	6.5
Mg^{2+}	1.0	1.5
Ca^{2+}	2.1-2.6	2.5
Cl^-	95.0-107.0	148
HCO_3^-	27.0	4.2
HPO_4^{2-}	0.65-1.45	1.0
SO_4^{2-}	1.0	0
pH	7.2-7.4	7.40

SBF was buffered with tris(hydroxymethyl)aminomethane and appropriate amount of hydrochloric acid.

Experimental methods

The crystal phase growth in the partially crystallized glass ceramics at different temperatures were determined by STOE Powder Diffraction System (STADI P) using CoK radiation with a wavelength of l = 1.788 nm, operating at 40 kV and 30 mA. Before the analysis, samples of each composition were milled into fine powder. All pattern were collected in the range of 10° to 60° 2Theta with a scan speed of 2°/min and step size 0.02. Combinations of time and temperature were used for nucleation and growth processing to produce different volume fractions of different crystallized phases. Surface microstructures of samples before and after immersion in SBF were examined by SEM (TESLA BS 300) after gold coating. 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 during exposure in SBF. The present paper reports the complete results of the influence of fluorapatite upon the bioactivity of glass ceramics in static regime and dynamic regime.



a) b)

Figure 1. a) Static regime: the glass ceramic sample is soaked in SBF; b) Dynamic regime: experimental construction (1 - inlet valve SBF, 2 - pump, 3 - pumping tank SBF, 4 - incubation apparatus, 5 - samples, 6 - closing valves, 7 - SBF).

RESULTS AND DISCUSSION

XRD results of glass ceramics

Figure 2a shows influence of P_2O_5 content upon the crystallization of glass ceramics with different content of P_2O_5 processed at 750°C. It is evident that that the crystallization of fluorapatite depends, not only on temperatures, but manly on P_2O_5 content. With increasing P_2O_5 content the ratio of fluorhydroxyaptite (FA) crystalline increased and peak intensity attributed to LS_2 ($Li_2O.2SiO_2$) decreased (Figure 2a). Crystalline phase of LS_2 is present in all samples thermally treated at 750°C. In samples containing 10 wt.% and 14 wt.% of P_2O_5 and heated at 750°C, crystalline FA appeared besides crystalline LS_2.

Figure 2b reports results of XRD analysis of two glass ceramics (sample with 14 wt.% of P_2O_5) processed at 550°C and 750°C. The XRD patterns show the presence of one or two crystalline phases depending on annealing temperatures. It can be seen on XRD patterns that lithium disilicate crystallizes in this system after heat treatment at 550°C (JCPDS 17-0447 with d = 3.67(100), 3.21(80), 3.50(60) and 3.60(2) Å), while fluorapatite remains in amorphous state. This fact is demonstrated by XRD pattern of sample containing 14 wt.% P_2O_5 showing more amorphous character (Figure 2b) at 550°C. Upon this finding it could be deduced, that P_2O_5 reacts with CaO to form $Ca_5(PO_4)_3F$ (FA), that is in amorphous form after heat treatment at 550°C and inhibits the crystallization of LS_2 [4]. XRD pattern of glass ceramics obtained at 750°C shows two distinct crystallized phases (lithium disilicate and fluorapatite). It can be concluded that lithium disilicate crystallizes firstly at lower temperatures, while fluorapatite crystals (JCPDS 15-0876 with d = 2.80(100), 2.77(55), 2.70(60), 2.62(30) Å) are formed at higher temperatures. Kuzielová et al. [4] have demonstrated that P_2O_5 at lower concentration acts as nuclear agent for lithium disilicate crystallization and promote it via surface mechanism.

THE RESULTS OF *IN VITRO* BIOACTIVITY TESTING

Surface analysis of glass ceramics before immersion in SBF

Figure 3a-e show SEM micrographs of glass ceramic samples heat-treated at 750°C before soaking in static SBF. The surface microstructure is composed of agglomerates of several micrometers, with smooth surface and changes with P_2O_5 conetnt in samples. The micro cracks observed on the surface of samples with higher content of P_2O_5 are due the partial crystallization. Indeed, authors [19, 20, 21] have demonstrated that the presence of fluorapatite supports the crystallization via surface mechanism.

The surface of glass ceramic samples with 0 wt.% and 14 wt.% of P_2O_5 and heat treated at 750°C was analysed by Microprobe (Table 3). 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.

The surface analysis by EPMA of sample containing 14 wt.% of P_2O_5 is depicted in Table 3. Also, one can note the dominant presence of SiO_2 on the surface. Next to SiO_2, calcium and phosphate are detected.

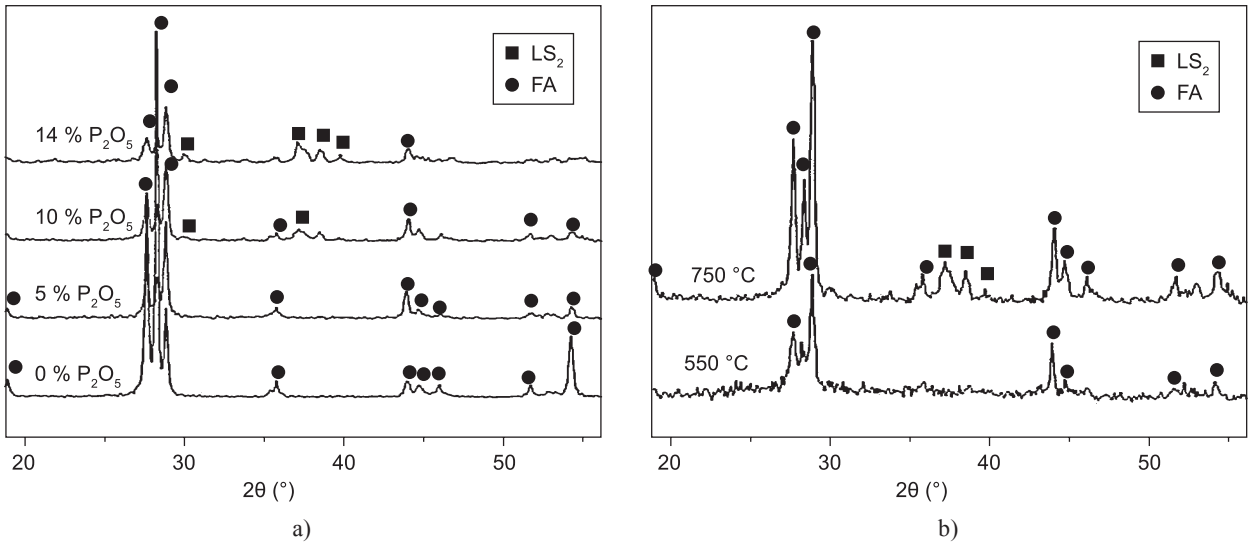
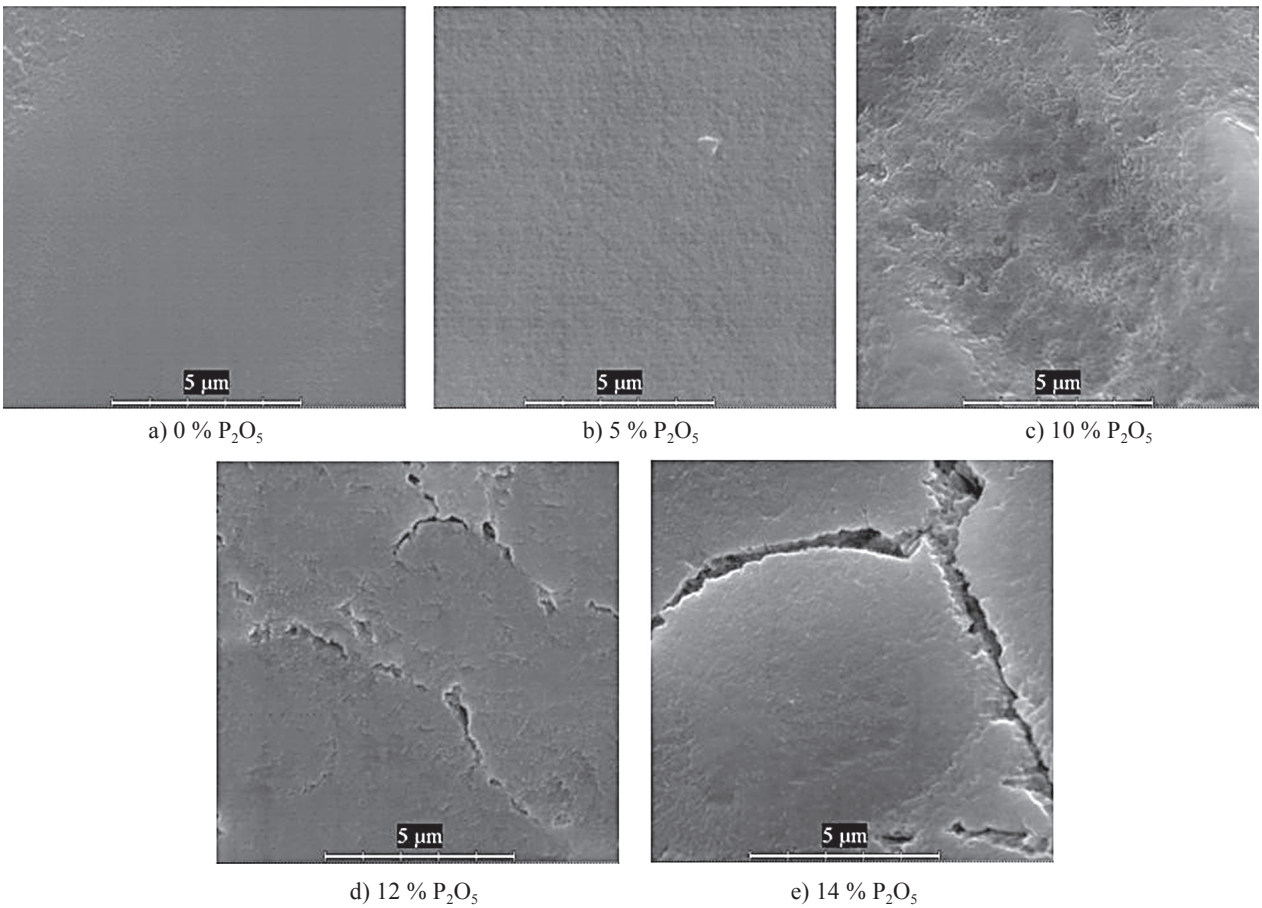


Figure 2. XRD diffraction patterns of samples: a) with different P_2O_5 content (wt.%) heated for 6 h at 750 °C; b) containing 14 wt.% P_2O_5 heated for 6 h at 550°C and 750°C.



a) 0 % P_2O_5 b) 5 % P_2O_5 c) 10 % P_2O_5

d) 12 % P_2O_5 e) 14 % P_2O_5

Figure 3. Microstructure of glass ceramics with different content of P_2O_5 before immersion in SBF.

Table 3. Amount of silicon, calcium and phosphorus present in the surface of glass ceramics without P_2O_5 and with 14 wt.% P_2O_5 before immersion in SBF (Analyzed by Electron Probe Micro Analyzer - EPMA).

Atomic %	0 wt.% P_2O_5	14 wt.% P_2O_5
Si	87.59	86.92
Ca	3.77	5.61
P	ND	4.50
Ca/P	–	1.25

ND - not detected

Surface analysis of glass ceramics after 4 weeks immersion in SBF

Figure 4a-e reports the bioactivity behaviour under static regime of glass ceramics with different content of fluorapatite. It is clear that the surface of different samples is covered by new layer with different thins. The layers increase with increasing content of fluorapatite in glass ceramics, demonstrating thus the influence of this compound upon the bioactivity of glass ceramics in Li_2O-SiO_2 system. Indeed, a continual and huge layer of new phases were created on the surface of the samples containing 10 wt.% P_2O_5, 12 wt.% P_2O_5 and 14 wt.% P_2O_5 (Figure 4c-e). In our previous work [22], the thickness of layer was determined according to P_2O_5 content. It varied from 272 nm to 1.69 µm for samples without content P_2O_5 and between 900 nm to 7.33 µm for samples with 14 wt.% P_2O_5. The micro cracks observed onto the surface are due to the drying process before SEM observation. Samples without P_2O_5 content and with 5 wt.% P_2O_5 show surface partially covered with dispersed regions of new phases (Figure 4a-b).

The surface analysis by Microprobe has proved that the new layer is composed of phosphate, calcium compound in proportion Ca/P = 1.66 similar in hydroxyapatite (Table 4). The mechanism and kinetics of hydroxyapatite formation during immersion in SBF is not until now full understood. The main theory postulates that functional groups of Si–OH are formed on the surface of glass and glass ceramics immediately after immersion in SBF solution by reaction of water with samples. These functional groups react with calcium ions dissolved in SBF to form Si–O–Ca bound with affinity towards phosphate ions. This complex process lead to the precipitation of hydroxyapatite on the surface of glass and glass ceramics from Li_2O–SiO_2 system.

Indeed, if P_2O_5, CaO and CaF_2 are added to base glass, fluorapatite is formed. The compound in amorphous or crystallized form act as scaffold for initiation of

hydroxyapatite precipitation because of their similar structure. Therefore, the thickness and the chemical composition of created layers varied in dependence on P_2O_5 content (Figure 4a-e). The formation of dense apatite layer, as characterised by local microanalyses (Table 4) and SEM micrographs, was evidently promoted by P_2O_5 addition. The significant growth occurred with higher P_2O_5 content (12 wt.%, 14 wt.%). This is due to the presence of fluorapatite in glass ceramics, which increases the rate of apatite layer formation and consequently enhances the bioactivity of samples in SBF. The surface roughness due to the crystallization supported by temperatures and P_2O_5 content can play a significant role in bioactivity of glass-ceramics.

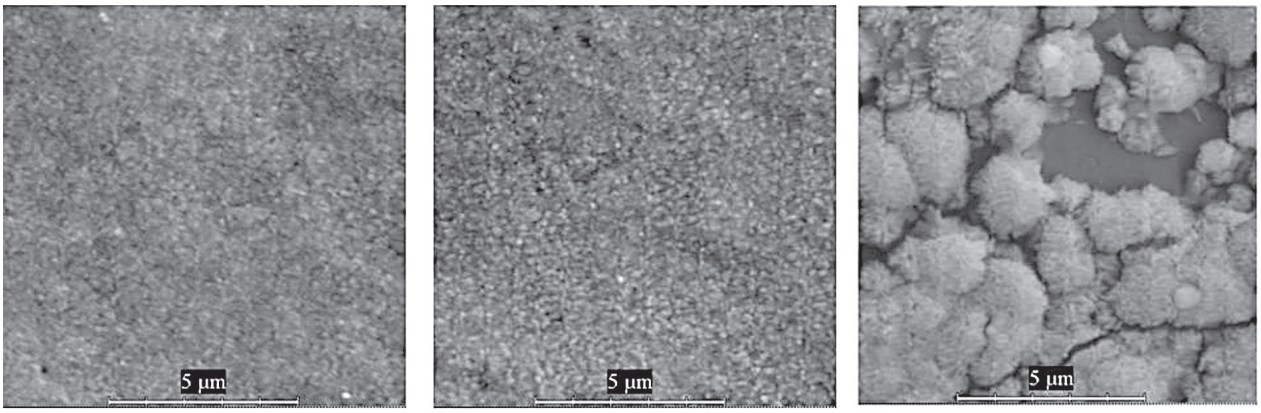
Table 4. Amount of silicon, calcium and phosphorus present in the surface of glass ceramics without P_2O_5 and with 14 wt.% P_2O_5 after 4 weeks immersion in static SBF (Analyzed by Electron Probe Micro Analyzer - EPMA).

Atomic %	0 wt.% P_2O_5	14 wt.% P_2O_5
Si	97.46	ND
Ca	0.33	59.87
P	1.39	36.14
Ca/P	0.24	1.66

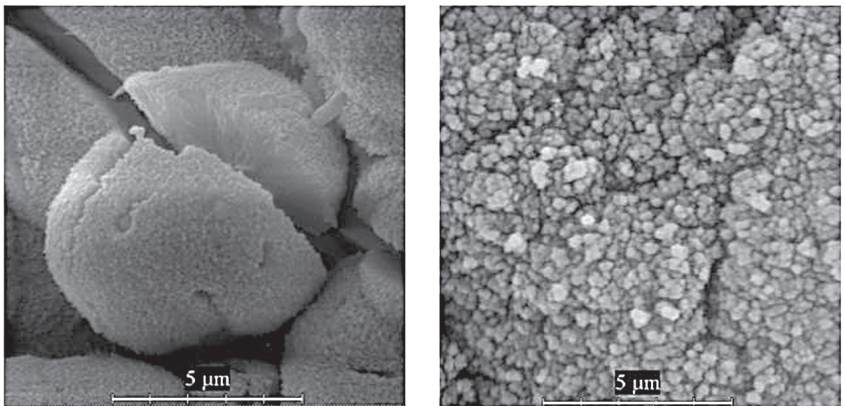
ND - not detected

On the contrary to results of surface analysis before dipping in SBF, a small amount of calcium and phosphorus besides silicon was detected on the surface of pure lithium disilicate glass ceramics after *in vitro* testing. The thickness of new layer is not too huge to cover the whole surface. The glass ceramic samples with higher P_2O_5 addition (10 wt.%, 12 wt.% and 14 wt.%) show high reactivity with the SBF by forming calcium phosphate-rich layers on their surfaces after 4 weeks of immersion. New created apatite layers cover whole initial surface and the microanalysis did not reveal any presence of silicon on these samples (Table 4). Besides these major elements the presence of small amount of Na, Mg and Cl was detected in all samples after immersion in SBF by EPMA. The above components were dissolved in simulated body fluid (see Table 2) and also are incorporated into the layer of hydroxyapatite. The Ca/P atomic ratio of new layer on the surface of glass ceramic samples is 1.66 indicating thus that of hydroxyapatite is the phase precipitated during immersion.

The preliminary results of bioactivity *in vitro* test under dynamic regime are depicted in Figure 5a-e. The microstructure differs from that of samples treated in static regime. One can not observe an important layer of hydroxyapatite on the surface in despite of higher P_2O_5 content. Nevertheless the formation of apatite layer



a) 0 % P_2O_5 b) 5 % P_2O_5 c) 10 % P_2O_5



d) 12 % P_2O_5 e) 14 % P_2O_5

Figure 4. Microstructure of glass ceramics with different content of P_2O_5 after 4 weeks immersion in SBF (static regime).

depends on P_2O_5 content. The main finding of this new test regime is that, microstructure of hydroxyapatite layer is finely ordered into porous structure and seems to be closer to the bone structure.

Table 5 reports the results of EPMA analysis of two samples without P_2O_5 and with 14 wt.% P_2O_5 after immersion in SBF under dynamic regime. The presence of silicium is negligible on surface but calcium, phosphorous and magnesium constituting the new phase were the main elements. The Ca/P atomic ratio of 1.29 is lower than that found in dynamic regime. This result is in accordance with those reported by [1These preliminary tests in dynamic regime let confirm that the mechanism and kinetics of apatite formation differ from that occurring under static regime. Indeed, the formation of new layer on surface of sample under static regime occurs by precipitation of hydroxyapatite from saturated solution of SBF. Under dynamic regime, apatite formation rate is partially inhibited by constant surface rinsing due to the circulation of SBF around the samples.

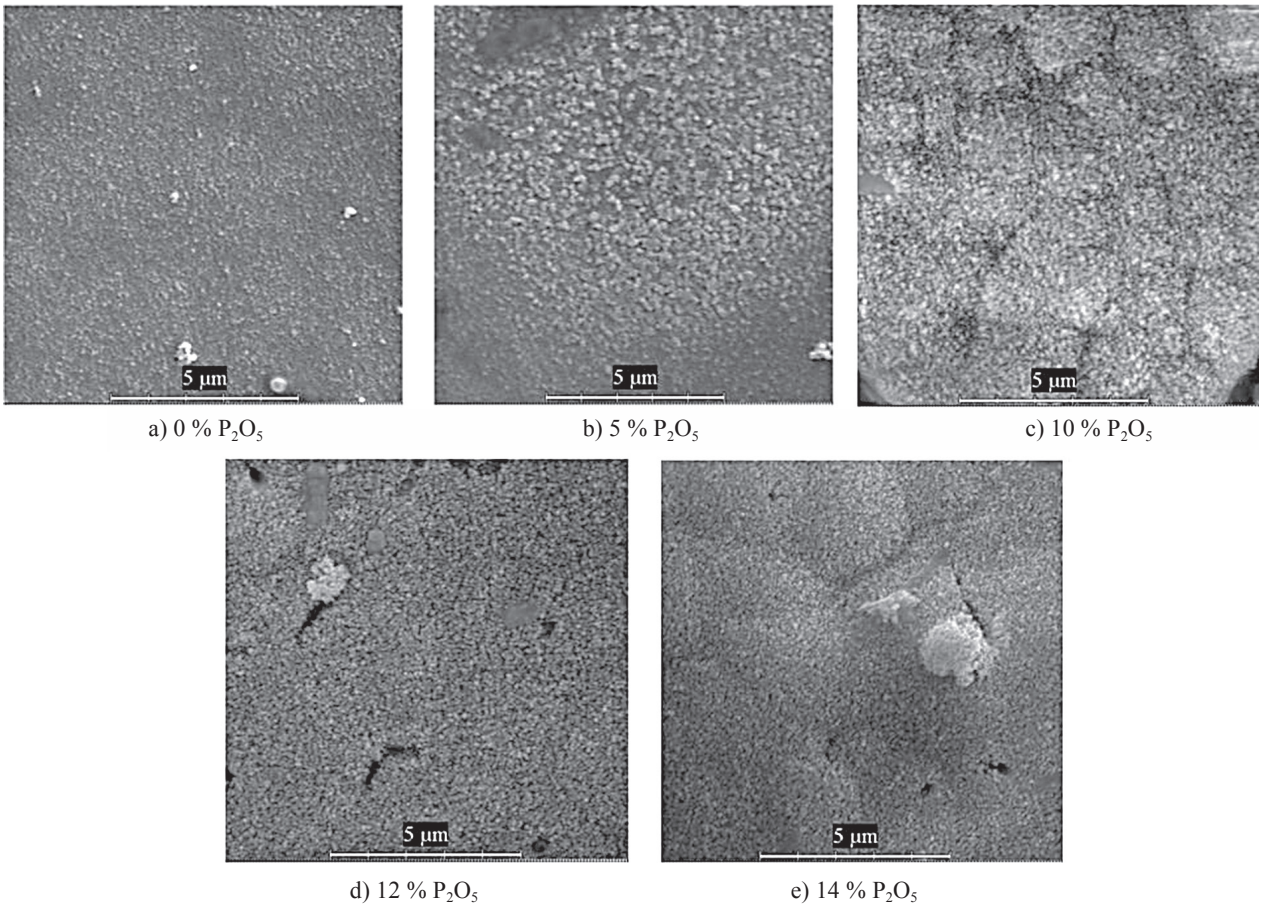
Table 5. Amount of silicon, calcium and phosphorus present in the surface of glass ceramics without P_2O_5 and with 14 wt.% P_2O_5 after 4 weeks immersion in dynamic SBF (Analyzed by Electron Probe Micro Analyzer - EPMA).

Atomic %	0 wt.% P_2O_5	14 wt.% P_2O_5
Si	95.68	ND
Ca	0.21	53.44
P	1.00	41.45
Ca/P	0.21	1.29

ND - not detected

CONCLUSION

The present work reports the results of *in vitro* test of bioactivity of glass ceramics with different content of fluorapatite expressed as P_2O_5. Two test regimes (static and dynamic) were applied to compare the mechanism and microstructure of apatite layer formed on the surface of samples after immersion in SBF. The presence of fluorapatite enhances the bioactivity of glass ceramics by formation of a dense layer of hydroxyapatite. Ca^{2+} resulting from the partial dissolution of glass sample at earlier period enhances the degree of supersaturation of SBF liquid in static regime. As SBF is supersatured with regard to Ca^{2+} and PO_4^{2-}, then hydroxyapatite precipitates massively at the beginning. In this case, the new layer of hydroxyapatite grows by chemical deposition of spherical apatite bullets. The Ca/P ratio is lower that than found in hydroxyapatite. The structure of apatite layer is



a) 0 % P_2O_5 b) 5 % P_2O_5 c) 10 % P_2O_5

d) 12 % P_2O_5 e) 14 % P_2O_5

Figure 5. Microstructure of glass ceramics with different content of P_2O_5 after 4 weeks immersion in SBF (dynamic regime).

very fine and bone like. In case of dynamic assays, where the liquid is circulating, the degree of supersaturation is not higher and the precipitation process is retarded. The microstructure is porous and apatite layers grow by continual reaction between them and fluid. The Ca/P ratio is the same in hydroxyapatite. In particular, it is evident that the bioactivity of glass ceramics is strongly influenced by P_2O_5 content in both regimes, but apatite-like formation in dynamic SBF is a better model than in static regime for it is more similar to the real condition of the body. This study has demonstrated that the mechanism and rate of apatite formation on the surface of glass ceramics differ according to test regimes.

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