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In vitro evaluation of bioactivity of SiO₂-CaO-P₂O₅-Na₂O-CaF₂-ZnO glass-ceramics

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Zinc is an essential trace element that stimulates bone formation but it is also known as an inhibitor of apatite crystal growth. In this work addition of ZnO to SiO₂–CaO–P₂O₅–Na₂O–CaF₂ glass-ceramic system was made by conventional meltquenching technique. DSC curves showed that the addition of ZnO moved the endothermic and exothermic peaks to lower temperatures. X-ray diffraction analysis did not reveal any additional phase caused by ZnO addition and showed the presence of wollastonite and hydroxyapatite crystalline phases only in all the glass-ceramic samples. As bio-implant apatite forming ability is an essential condition, the surface reactivity of the prepared glass-ceramic specimens was studied in vitro in Kokubo's simulated body fluid (SBF) [1] with ion concentration nearly equal to human blood plasma for 30 days at 37 °C under static condition. Atomic absorption spectroscopy (AAS) was used to study the changes in element concentrations in soaking solutions and XRD, FT-IR and SEM were used to elucidate surface properties of prepared glass-ceramics, which confirmed the formation of HCAp on the surface of all glass-ceramics. It was found that the addition of ZnO had a positive effect on bioactivity of glassceramics and made it a potential candidate for restoration of damaged bones.

Keywords: bioactive glass ceramics; in vitro bioactivity; apatite layer; zinc oxide

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1. Introduction

Glass-ceramics is a fine grained polycrystalline material prepared by controlled heat treatment. Glass-ceramics have been widely used as bone grafts or fillers for orthopaedic and dental applications. The most useful property of glass-ceramics, which shows the ability of the material to form a direct bond to a living bone, is bioactivity [2]. Bioactivity is a spontaneous communication of materials in biological environment, which results in strong adhesion between tissue-implant interface [3].

Zinc is an essential trace element [2, 4–7] whose addition promotes bone formation as it stimulates osteoblast proliferation and differentiation [8]. In addition to that it has profound antibacterial properties [9, 10]. ZnO increases the tendency towards crystalline phase separation as it decreases the viscosity of glass. Zinc oxide is known

to be a potent inhibitor of crystal growth of apatite phase; it reduces the dissolution rate, thus improving chemical stability of glass [4]. An increase in concentration of ZnO in glass and glass-ceramic system causes decline in apatite forming ability. The amount of ZnO to a base system, which may lower the bioactivity level, was defined as 5 % by S. M. Salman et al. [3] while M. Kamitakahara et al. [11] reported more than 0.7 % ZnO and A.K. Srivastava and R. Pyare 2012 findings suggested that more than 1 % ZnO content to base system lowered the bioactivity level. In their study Shirong Ni et al. [12] found that Na increases the bioactivity of 58S bioactive glass as it provides faster dissolution rate; so a small amount of Na₂O was added to SiO₂-CaO-P₂O₅-CaF₂ system to improve its ionic activity in simulated body fluid.

This study deals with the effect of ZnO on the structural and biological properties of the system. The influence of Zn additive on the deposition of bone-like apatite layer on the surface of a sample

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Sample id:	Composition (mol %)						
Sample Id.	SiO_2	CaO	$P_2O_5\\$	ZnO	Na ₂ O	CaF_2	
BZn0	38.73	49.96	6.53	0	4.35	0.43	
BZn0.76	38.73	49.20	6.53	0.76	4.35	0.43	
BZn3.63	38.73	46.33	6.53	3.63	4.35	0.43	
BZn7.78	38.73	42.18	6.53	7.78	4.35	0.43	
BZn15.53	38.73	34.43	6.53	15.53	4.35	0.43	

Table 1. The chemical composition of samples.

in simulated body fluid was investigated and its effect on thermal and structural properties of a glassceramic system was also evaluated in present work.

2. Materials and methods

2.1. Glass preparation

Glass was prepared from a composition of (38.73)SiO₂·(49.96 - x)CaO·(x)ZnO·(6.53)P₂O₅· (4.35)Na₂O·(0.43)CaF₂ {x = 0, 0.76, 3.63, 7.78, 15.53 mol %} using chemically pure (99.9 %) silicon dioxide (SiO_2) , calcium oxide (CaO), sodium carbonate (Na_2CO_3) , phosphorous pentaoxide (P_2O_5) , calcium fluoride (CaF_2) and zinc oxide (ZnO); (see Table 1). The weighted batches were homogeneously mixed using an agate mortar and a pestle before melting in a Pt crucible for 2 h in an electric furnace at 1450 °C temperature. and the melts were rotated two times to achieve homogeneity. The melts were poured on a stainless steel plate and then milled in an agate mortar with a pestle and sieved through a mesh screen with the mesh size of 40 micron. The powdered glass was then pressed into discs of 15 mm diameter under hydrostatic pressure of 48 MPa.

2.2. Differential scanning calorimetric analysis (DSC)

DSC measurements (SDT Q600 V8.2 Build 100) were carried out on fine powdered glass samples examined in temperature range from room temperature to 1000 °C at 15 °C/min rate using alumina as a reference material.

2.3. Preparation of glass-ceramic samples (heat treatment regime)

All the glasses were converted into glassceramics by controlled two-step heat treatment. The thermal data obtained from DSC curves provided the heat treatment schedule for the samples, which consisted of two step regime, the first temperature was equivalent to the nucleation step and the second temperature was equivalent to the maximum crystal growth step, and these two temperatures were correlated with endothermic and exothermic peaks of the DSC thermogram. For all samples, the time of holding at the first nucleation temperature was taken as 2 h to ensure maximum nucleation, and the time of holding at the crystallization step was taken as 4 h to ensure maximum crystal growth. After two-step heat treatment the samples were left overnight to cool inside the electric furnace to room temperature.

2.4. Characterization of glass-ceramic samples

The characterization of glass-ceramic samples was carried out using BRUKER AXS D8Discover X-ray diffractometer, using CuK α radiation in 2θ range of 20° to 40° to identify the precipitated crystalline phases before and after soaking in SBF solution. The International Center for Diffraction Data cards were used as the reference data for interpretation of XRD patterns. The surface texture of prepared glass-ceramic samples was examined before and after immersion in SBF with scanning electron microscope (SEM) JEOL model JSM-6480LV.

2.5. In vitro bioactivity analysis

The assessment of in vitro bioactivity was carried out in simulated body fluid SBF with ion concentration similar to human blood plasma, mentioned in Table 2.

The SBF solution was prepared by dissolving reagent grade chemicals one by one, in the order mentioned in Table 3, in distilled water. The SBF solution was buffered to a pH value of 7.4 with Tris (hydroxymethyl) aminomethane (NH₂C(CH₂OH)₃) and 1M hydrochloric acid (HCL) at 37 °C.

	Na ⁺	K^+	Mg^{2+}	Ca^{2+}	Cl^{-}	HCO_3^-	HPO_4^{2-}	SO_4^{2-}
SBF	142.0	5.0	1.5	2.5	147.8	4.2	1.0	0.5
Blood plasma	142.0	5.0	1.5	2.5	103.0	27.0	1.0	0.5

Table 2. Ion concentration (mM) in SBF and human blood plasma.

Table 3. Reagents of updated simulated body fluid (reagent amount for 1L of SBF) [10].

Sr. No.	Reagent	Amount
1.	sodium chloride (Merck, Germany)	8.035 g
2.	sodium bicarbonate (Merck, Germany)	0.355 g
3.	potassium chloride (Merck, Germany)	0.225 g
4.	potassium phosphate dibasic trihydrate (Merck, Germany)	0.231 g
5.	magnesium chloride hexahydrate (Merck, Germany)	0.311 g
6.	1 M hydrochloric acid	39 mL
7.	calcium chloride (Merck, Germany)	0.292 g
8.	sodium sulphate (Merck, Germany)	0.072 g
9.	tris(hydroxymethyl) aminomethane (Fluka, UK)	6.118 g

All the glass-ceramic samples were soaked in above mentioned SBF under static condition at 37 °C for different time periods 1, 7, 21, 24 and 30 days. A surface area to volume ratio of 0.1 cm^{-1} was maintained for all immersions.

The surface structure of glass-ceramic samples removed from SBF solution after each time interval was examined by MIDAC, M series Fourier transform infrared spectrometer, with IR spectra recorded in the spectral range of 4000 to 400 cm^{-1} at 16 cm⁻¹ resolution with reference to KBr. The elemental concentration of SBF solution for each time period was measured with Polarized Zeeman Atomic Absorption Spectrophotometer Z-5000, Hitachi. The variation in pH value of SBF solution was recorded by pH meter (model AD1011, Adwa).

3. Results and discussion

3.1. Differential scanning calorimetric analysis

Fig. 1 shows DSC curves and Table 4 displays glass transition temperature T_g and crystallization temperature T_c of the base and the above mentioned glasses. The glasses were converted into ce-

ramics derivative by two step heat treatment. In the first step BZn0, BZn0.76, BZn3.63, BZn7.78 and BZn15.53 glasses were heated at 740 °C, 722 °C, 712 °C, 701 °C and 654 °C ($\sim T_g + 40$ °C), respectively, for two hours in order to obtain desired nucleation in the glass. Thereafter, all the samples were sintered for four hours at T_{c2} crystallization temperature for optimum crystal growth. It can be observed that the substitution of CaO with ZnO caused some shift of both exothermic and endothermic peaks to lower temperatures. The nucleation temperature at 740 °C and crystallization temperature at 910 °C for the parent glass (BZnO) was reduced to 654 °C and 746 °C, respectively, after 15.53 mol % substitution of ZnO into the system. This result can be attributed to the decrease in viscosity of the system with the increase of ZnO as reported by Doremus [14] and Alizadeh et al. [15], Kamitakahara et al. [11], Mirhadi et al. [16] and Srivastar and Pyare [7].

It was seen that when the substitution of ZnO content increased above 0.76 mol % broadening of exothermic peaks was observed, which might be due to overlapping of the peaks. It was also found that when ZnO content was raised to 15.35 mol % the exothermic peaks merged into a single broad peak. It was also observed that no sharp exothermic

Sample ID Glass Transition temperature Crystallization temperature Crystallization temperature					
	\mathbf{T}_{g} (°C)	\mathbf{T}_{c1} (°C)	\mathbf{T}_{c2} (°C)		
BZn0	700	856	910		
BZn0.76	682	847	917		
BZn3.63	672	815	897		
BZn7.78	661	N.A	785		
BZn15.53	614	N.A	746		

Table 4. Glass transition temperature T_g and crystallization temperature T_c .

N.A represents no peak observed



Fig. 1. The DSC thermogram of glass-ceramics showing crystallization temperature T_c .

effect occurred for BZn7.76 and BZn15.53 glass in the DSC curves; it means that the amount of crystalline phases developed during DSC run was small, which is consistent with XRD analysis shown in Fig. 2.

3.2. Structural characterization

Fig. 2 shows XRD patterns of the surfaces of the glass-ceramics, which reveal the presence of two crystalline phases: hydroxyapatite (PDF#00-074-0566) and wollastonite (PDF#00-029-0372). However, addition of ZnO \leq 3.63 mol % into the system increased the intensity of the peaks of wollastonite phase, but higher substitution of zinc oxide at expense of calcium oxide restrained the growth of wollastonite crystals and wollastonite phase was completely decomposed at 15.53 mol % addition of ZnO, while the hydroxyapatite phase was initially slightly reduced due to the substitution of CaO with

ZnO but afterwards it has not varied much. As no apparent peak shift was observed it may be assumed that ZnO remained in the glassy phase, as no distinct crystalline phase was detected. It was also observed that ZnO reduced the crystallization ability of the system for \geq 7.76 mol % addition to the base system.



Fig. 2. X-ray diffraction of (a) BZn0, (b) BZn0.76, (c) BZn3.63, (d) BZn7.78, and (e) BZn15.53 glass-ceramics.

3.3. In vitro dissolution test

3.3.1. FT-IR analysis

The FT-IR analysis shown in Fig. 3 and 4 represents changes in surface composition after soaking in simulated body fluid. The IR transmittance bands for BZn0 glass-ceramics before immersion in the SBF solution reveal sharp peaks at silicate transmission bands at about 480.7, 799.4,

717.3, 942.9, 1086 cm⁻¹, phosphate transmittance at 568.3 cm^{-1} , and hydroxyl bands at about 1639.38 cm⁻¹ and 3454.2 cm⁻¹. This IR transmittance spectra peaks before immersion in SBF reveal the presence of Si-O-Si bending (500 to 400 cm^{-1}), Si-O-Si stretching (940 to 860 cm⁻¹) and Si–O–Si tetrahedral (1175 to 710 cm^{-1}) bands, which are well accepted to be the characteristic of the silicate network. This may be interpreted as the presence of silica as a basic building constituent. No separate phosphate band was observed in the FT-IR spectra, which may be due to the limited amount of P₂O₅ present in the system but hydroxyl band (1700 to 1600 cm⁻¹) and (3600 to - 3200 cm^{-1}) appeared due to the presence of hydroxyapatite phase [6, 7, 17–21].

The following changes were observed after various reaction times:

After 1 day: the silicate bands at 480.7 cm^{-1} , 1086 cm⁻¹ and 942.9 cm⁻¹ have been shifted to 494.9 cm⁻¹, 1108.5 cm⁻¹ and 1065.9 cm⁻¹, respectively; the two silicate bands at 717.3 cm⁻¹ and 799.4 cm⁻¹ disappeared and two new phosphorous bands 536.7 cm⁻¹ and 607.7 cm⁻¹ appeared.

After 7 days: almost similar to IR spectra observed after 1 day with slight shift in silicate bands at 494.95 cm⁻¹ and 1065.9 cm⁻¹ to 509.2 cm⁻¹ and 1147.2 cm⁻¹, respectively, along with the appearance of a new amorphous P–O band at around 558 cm⁻¹.

After 21 days: the silicate (Si–O–Si tetrahedral vibration mode) band at 1147.2 cm⁻¹ disappeared and P–O band at 1101.47 cm⁻¹ emerged.

After 24 days: the appearance of two new transmission bands, the carbonate bands at 1404.3 cm⁻¹ and 800.9 cm⁻¹ and the phosphorous (P–O bend/crystal) band at 589.6 cm⁻¹.

After 30 days: the appearance of a sharp and intense P–O band at 1101.47 cm⁻¹, the carbonate bands at 1394.09 cm⁻¹ and 800.9 cm⁻¹, phosphorous crystal band at 611.68 cm⁻¹ and hydroxyl bands at around 1639.38 cm⁻¹ and 3454 cm⁻¹ indicate formation of hydroxycarbonate apatite crystalline layer.



Fig. 3. FT-IR transmittance spectra of BZn0 glassceramics before and after immersion in SBF at different intervals between 1 to 30 days.



Fig. 4. Comparative IR transmittance spectra for all glass-ceramic samples after 30 days.

The IR spectra (Fig. 4, Table 5) obtained after soaking in SBF for 30 days show that the bands assigned to P–O vibrations, which are characteristic of HA crystalline phase, can be detected for all the glass-ceramic samples. The emergence of a new band characteristic of a typical C–O vibration and the presence of two P–O and H–O bands clearly indicate the formation of a crystallized hydroxyl-carbonated apatite (HCA) layer in these samples [13, 18]. The band of Si-O stretch has disappeared only for BZn0, but silicate bands are still present, though weak, for other samples. It may attributed to polymerization of the Si-OH groups formed at the initial stages by exchange of hydrogen ions from the solution containing alkali ions [13]. FT-IR studies clearly indicate that all glass ceramic samples show bone bonding ability. The addition of ZnO has not decreased the formation of HCA layer, in contrary to the results by Srivastava and Pyare [7] where the addition of >1 % ZnO into the system decreased the formation of the apatite layer. In our study it was found that increasing the concentration of ZnO (particularly $>3.63 \mod \%$) in the system predominantly reduced the degree of crystallization of the system which was confirmed by XRD (Fig. 2) and SEM (Fig. 7). This less densified or less crystalline structure favoured in vitro bioactivity of the material, as the residual glassy phase played a major role in enhancing the ionic activity of the material.

3.3.2. XRD analysis

The XRD patterns of the surfaces of the glassceramics after immersion in SBF for 30 days, shown in Fig. 5, confirm the formation of (HCAp) carbonated hydroxyapatite (PDF#04-0697) crystalline phase in all glass ceramic specimens, while the wollastonite phase disappears. It can be seen that with the increase in concentration of ZnO in the system the apatite formed in the physiological environment on the surface of the samples shows an increase in intensity of the peaks. This sharpening of peaks is an indicator of good apatite crystal symmetry. The formation of apatite layer has also been confirmed by SEM images (Fig. 8). Thus, the addition of ZnO shows a positive effect on in vitro bioactivity of the system.

3.3.3. AAS analysis

The change in pH and Si, Ca, P, Zn and Na element concentrations in SBF solution recorded throughout the length of immersion period is shown in Fig. 6. The variation of pH of SBF solutions is consistent with the mechanism of nucleation proposed by Hench et al. [22] and the growth



Fig. 5. The XRD patterns for (a) BZn0, (b) BZn0.76, (c) BZn3.63, (d) BZn7.78, and (e) BZn15.53 glass-ceramics after 30 days immersion in simulated body fluid (SBF).

of bone-like apatite layer on the surface of bioactive materials, but at the very initial stage a slight decrease in pH of solution was also observed for all glass-ceramic samples except the BZn0. This decrease can be understood by taking into account the initial fast release of zinc ions to SBF solution, which changes the solution acidity [2, 23]. It was also found that the enhanced release of zinc ions causes simultaneous decrease of both pH of the solution and amount of Si ions leached out from the sample as ZnO has been known as an amphoteric oxide that improves chemical durability by reducing the dissolution rate of glass-ceramic samples by decreasing the number of non-bridging oxygens [3, 11].

Hench et al. [22] proposed that the initial increase in pH of the SBF resulted from the rapid exchange of Na⁺ and Ca²⁺ ions of glass-ceramics with H⁺ and H₃O⁺ ions from the solution. Then, the pH of the solution started to decrease as the leaching process ceased and the need for hydrogen ions was reduced. A gradual increase of Si with a slight decline in the P content was observed with the length of soaking. Ca concentration increased during 15 days of soaking in SBF and then decreased gradually; this increase may be attributed to the release of calcium ions from the glass-ceramics while the decrease in



Fig. 6. Variation of Ca, P, Si, Na, Zn concentration and pH of the SBF after soaking for various time intervals.

Group	Group Wave number (cm ⁻¹)			After 30 days immersion in SBF					
and vibration	literature [6, 7, 17–21]	BZN0	BZn0.76	BZn3.63	BZn7.78	BZn15.53			
H–O–H stretch	3550	34540	3451.05	3477.87	3488.12	3462.8			
H–O stretch	1700 - 1600	1639.38	1625.18	1648.84	1634.64	1644.9			
C–O stretch	1460 - 1415	1394.09	1409 - 1470.6	1404.5	1402.7	1405.1			
P=O stretch	1350 - 1080	1101.47	1083.3	1101.47	1094.37	1097.53			
Si-O stretch	1100 - 1000	_	—	_	_	_			
Si–O–Si stretch (2NBO)	1085 - 800	_	1018.6	_	1039.9	1031.28			
C–O stretch	890 - 800	800.9	781.2	790.8	796.2	799.4			
Si-O-Si sym stretch tetra	1175 – 710	_	—	954.6	_	_			
P–O bend/crystal	610 - 600	611.68	606.9	_	617.9	_			
P–O bend/amorphous	600 - 560	_	—	570	571.4	571.46			
P–O bend/crystal	560 - 500	_	—	491.02	495.7	564.36			

Table 5. The FT-IR spectra vibration modes for all glass ceramics after 30 days soaking in SBF.

Ca concentration after some days was due to the formation of CaO–P₂O₅ layer. The decrease in P concentration with a simultaneous increase in Si concentration is consistent with the formation of CaO–P₂O₅ layer [22]. It was observed that with the increase in concentration of ZnO in the system the amount of ionic dissolution decreased since ZnO enhanced the chemical durability of the material, but it had an insignificant effect on the formation of apatite. Addition of ZnO did not delay the apatite forming process; all samples started to deposit apatite after 15 days, as indicated by the decrease in Ca ion concentration.

3.3.4. SEM analysis

Fig. 7 and 8 show SEM images obtained from the glass-ceramic samples before and after soaking in SBF solution. Fig. 7 shows that glass-ceramic surfaces generally exhibit regular textures without pores, while Fig. 8 shows that after soaking in SBF solution for 30 days the precipitates of apatite have fully covered the surface of glass-ceramics BZn0, BZn0.76, BZn3.63 and BZn7.78. However, some scattered crystals of apatite can be observed on the surface of BZn15.53 glass-ceramics. The glass ceramics containing 0.76 to 15.53 mol % ZnO displays the capability of bonding with a human bone. Thus, an increase in ZnO content did not have a significant effect on the dissolution rate, which would improve the bioactivity of glass ceramics. Our findings are in contrary to Kamitakahara et al. [11] who reported that ZnO addition reduced bioactivity of glass ceramics and even the ZnO content as low as 0.7 mol % to SiO₂-CaO-P₂O₅-CaF₂ glass-ceramic svstem inhibited the growth of crystalline apatite layer. Based on our study and the XRD analysis, which confirmed the enhancement of apatite phase, we can conclude that addition of ZnO facilitates apatite formation in SiO₂-CaO-P₂O₅-Na₂O-CaF₂ based glass ceramics as it optimizes the atomic structure of the material.

4. Conclusions

We have synthesized glass ceramics in $SiO_2-CaO-P_2O_5-Na_2O-CaF_2$ system with partial substitution of ZnO with CaO containing apatite and wollastonite phases. The DSC thermal analysis curves revealed that the effect of introduction of ZnO in place of CaO caused a decrease of both glass transition temperature (T_g) and crystallization temperature (T_c). In vitro bioactivity studies on $SiO_2-CaO-P_2O_5-Na_2O-CaF_2$ glass-ceramics containing 0.76 to 15.53 mol % ZnO indicated that these materials had the capability of bonding with the human bone. FT-IR analysis demonstrated





(e)

Fig. 7. SEM micrographs of surfaces of glass-ceramics before soaking in SBF: (a) BZn0; (b) BZn0.76; (c) BZn3.63; (d) BZn7.78; (e) BZn15.53.





(d)



⁽e)

Fig. 8. SEM micrographs of surfaces of glass-ceramics after soaking in SBF for 30 days: (a) BZn0 30 days; (b) BZn0.76 30 days; (c) BZn3.63 30 days; (d) BZn7.78 30 days; (e) BZn15.53 30 days.

the characteristic HCA functional groups (PO_4^{3-} , CO_3^{2-} and OH^- groups), which indicated the presence of hydroxycarbonate apatite layer on the surface of SBF treated sample. The deposition of crystalline apatite layer on the surface of glass ceramics soaked in SBF was confirmed by XRD and SEM. It may be inferred from the results of this study that addition of ZnO facilitated apatite formation in SiO₂–CaO–P₂O₅–Na₂O–CaF₂ based glass ceramics and increasing concentration did not diminish the bone bonding ability of the glass-ceramics. In addition, it has been found that these glass-ceramics have predominant antibacterial properties, which make them the potential candidates for bone graft.

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