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Influence of fluoride for enhancing bioactivity onto phosphate based glasses

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ABSTRACT

Phosphate based glasses $48P_2O_5$—(32—*x*)CaO—$20Na_2O$—*x*CaF_2 (*x* = 0, 1, 2, 3 and 4 mol%, hereafter, termed as FPG0, FPG1, FPG2, FPG3 and FPG4 respectively) with different fluoride content were prepared by keeping the ratio of P/Ca as 3. To study the influence of fluoride on the physico-chemical properties and bioactivity of phosphate based glasses, various experimental analysis such as density, elastic moduli, micro hardness, X-ray diffraction, scanning electron microscopy, Energy dispersive X-ray spectroscopy, cytotoxicity, pH variations during *in vitro* studies by soaking in simulated body fluid as a function of fluoride content were carried out. A variation in density, micro hardness and elastic moduli is noted for the glass samples as a function of its fluoride content. X-ray diffraction pattern, Fourier Transform Infra-Red spectra, scanning electron microscope images and energy dispersed X-ray spectra confirmed the fluoroapatite forming ability on all the fluoride added glass samples. The elastic moduli, microhardness, rich formation of FAp and non-toxic nature of glass sample FPG3 showed the better bioactivity than all the other samples. The observed cell growth of human osteoblast like cell lines proved the non-toxic nature of glass sample FPG3. Thus the observed results confirms the glass sample with 3 mol% of CaF_2 content is suitable for implant applications.

1. Introduction

The search for suitable alternate material for the replacement of damaged and diseased bone has been done for the past three decades. Bioactive glass is the well-known synthetic bone graft material for different biomedical applications due to its better bone bonding ability, controlled solubility and improved mechanical strength [1,2]. A wide range of studies on silica based bioglasses supports to develop an alternate bone grafting material which should exhibit osteoconduction, osteoinduction and osteogenesis with equivalent mechanical strength as that of natural bone [3,4]. Silica free phosphate based glasses (PBGs) play an important role in different biomedical applications due to its tailor made solubility and low melting temperature while compared with silicate glasses [5]. The PO_4^{3+} tetrahedral structure in the PBG supports to connect with the metal ions which results the increase in mechanical strength and neutralize the inorganic phosphate chain [6]. The release of ca^{2+} and Na^+ ions in the PBG glass shows the potential applications as a synthetic bone graft material, since these ions are the natural constituents as that bone. Further, controlled degradability, solubility and higher dissolution rate of PBG opens a wide range of studies in bone tissue engineering [7].

Generally, fluoride is the promising material for inducing bone growth in the human body in physiological solutions [8]. Fluoroapatite (FAp) showed its higher rate of osteoconduction, lesser solubility and better chemical stability than crystalline Hydroxyapatite (HAp) [9]. Bioactive glass containing FAp as the crystalline phase is of great interest for both dental and orthopedic applications. FAp glass-ceramics shown to confer osseointegration and osteoconduction during clinical examinations on osteoporotic patients which confirms the fluoride treatment stimulates the bone formation [10]. Due to the corrosion inhibition nature of F^- ions, the chemical reactivity will decrease on the glass surface and to promote the formation of thin surface layer due to the fluoride content in bioactive glasses [11,12]. Lowering the glass transition temperature, better chemical durability and the formation of fluorine complexes are the notable properties of fluorophosphate glasses for different biomedical application.

Fluoride shows its toxic nature when consumed in higher concentrations. On the other hand, it shows beneficial changes in the body when the intake limit is up to 5% [13,14]. *In vitro* studies on the effect of fluoride on the cell responses have been extensively analysed in cell biology using culture medium [15–17]. Clifford et al. reported that fluoride is the promising material for inducing bone growth in human

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Table 1
Longitudinal (L), Shear (G), Young's (Y), Bulk (K) modulus, density (ρ) and Microhardness (H) along with the composition of fluorophosphate glasses at 303 K.

Sample code	Glass composition (mol%)				Density (kgm^{-3})	Micro-hardness	Modulus (GPa)			
							Longitudinal	Shear	Young's	Bulk
	P_2O_5	CaO	Na_2O	CaF_2	ρ	H	L	G	Y	K
FPG0	48 (69.42)*	32 (17.96)*	20 (12.63)*	0 (0)	2718 ± 1	2.56 ± 0.02	74.18 ± 0.02	20.32 ± 0.01	53.30 ± 0.03	47.09 ± 0.03
FPG1	48 (69.25)*	31 (17.35)*	20 (12.60)*	1.00 (0.79)*	2662 ± 4	2.39 ± 0.03	67.14 ± 0.01	18.63 ± 0.03	48.74 ± 0.04	42.30 ± 0.04
FPG2	48 (69.09)*	30 (16.76)*	20 (12.57)*	2.00 (1.58)*	2690 ± 2	2.13 ± 0.04	66.92 ± 0.05	17.71 ± 0.04	46.76 ± 0.03	43.31 ± 0.05
FPG3	48 (68.93)*	29 (16.16)*	20 (12.54)*	3.00 (2.37)*	2701 ± 3	2.08 ± 0.01	66.90 ± 0.02	17.54 ± 0.03	46.39 ± 0.02	43.51 ± 0.02
FPG6	48 (68.77)*	28 (15.57)*	20 (12.51)*	4.00 (3.15)*	2708 ± 3	2.06 ± 0.02	67.16 ± 0.01	17.50 ± 0.02	46.34 ± 0.04	43.82 ± 0.04

* The compositions in wt% is inside the bracket.

body. Further, the addition of fluoride in PBG system will improve the bone bonding ability than the fluoride free glasses due to the rich formation of FAp layer in biological fluid. Fluoride stimulates the growth of osteoblast cells when applied at moderate concentrations on cell cultures of osteoblasts (25–500 $\mu g ml^{-1}$) whereas higher concentrations (< 500 $\mu g ml^{-1}$) suppress osteoblastic activity [18]. Literature review shows that CaF_2 can be used as nucleating agent in phosphate glass system which leads the rich formation of FAp layer. The present paper deals with the effects of CaF_2 as nucleating agent in P_2O_5-CaO-Na_2O glass system for the improvement of bioactivity and apatite forming ability. The purpose of this study is to examine fluoride-containing bioactive glasses and the effects of fluoride incorporation on glass structure, mechanical properties and bioactivity. Moreover, the cytotoxicity was assessed on CaF_2 added glass samples using osteoblast-like cells (MG63). For that, five compositions $48P_2O_5$—(32—x)CaO—$20Na_2O$—xCaF_2 (where x = 0, 1, 2, 3 and 4 mol %) of glasses in different CaF_2 content are prepared, analysed and optimised.

2. Materials and methods

2.1. Preparation of glass samples

The $48P_2O_5$—(32—*x*)CaO—$20Na_2O$—*x*CaF_2 (*x* = 0, 1, 2, 3 and 4 mol%) glass of different compositions were prepared using commercially available chemicals by normal melt quenching method [19]. The Chemical Components included $NH_4H_2PO_4.2H_2O$ (99.999%), $CaCO_3$ (99.995%), Na_2CO_3 (99.9%) and CaF_2 (99%), which were of analytical grade (Sigma-Aldrich, Japan) and used without any further purification. The exact weight of each chemical reagent was measured using a digital balance (model BSA224S-CW; Sartorius, Goettingen, Germany) and ground using a ball mill (model PM 100; Retch, Haan, Germany) with zirconium balls of 10 mm dia. The mixture was melted in platinum crucible (10% Rhodium doped) for 1 h at 1323 K in an electric furnace. The homogenized melt was recast in a preheated stainless steel mould of rectangular shape with a size of 90 × 60 × 20 mm glass sample. The prepared glass sample was annealed at 573 K for 1 h in an electric furnace and cooled to room temperature at a rate of 1 K per minute. Specimen was cut from the prepared glasses using a diamond saw for various characterisation studies.

2.2. Characterisation

Physico-chemical properties were observed to assess the mechanical strength and apatite forming ability for all the prepared glass samples. The well-known characterisation studies such as density of the prepared fluorophosphate glass samples were obtained using Archimede's principle [20]. Elastic moduli of all the prepared glass samples were calculated by longitudinal and shear ultrasonic velocity measurements using ultrasonic process control system (model FUII050; Fallon Ultrasonics Inc. Ltd., Ontario, Canada), with a 100 MHz digital storage oscilloscope (model 54600B; Hewlett Packard, Palo Alto, California, U.S.A.) by adopting pulse echo method. Energy dispersive X-ray spectroscopy (model: X-mas 50 mm^2 Oxford Abingdon, England) was used to determine the experimental composition of prepared glass samples. X-ray photoelectron spectrometer (model Kratos; Ultra Axis, Manchester, UK) with Al and Mg Kα dual source operating at 210 W was used to conduct XPS studies [21]. The SBF solution was prepared in the laboratory using the method formulated by Kokubo et al. [22,23]. *In vitro* studies for all the prepared glass samples were done by soaking in SBF solution for 21 days at 310 K in a Biological Oxygen Demand (BOD) incubator. pH variations in SBF solution were noted for all 21 days using a pH meter (model Star A211; Thermo Orion, USA). All the measurements such as density, ultrasonic velocity, pH variation during *in vitro* studies *etc.* were taken in five times and the average values were used for calculation. The relative standard deviation (RSD) in the calculated results is shown in Table 1. X-ray diffraction pattern (XRD) pattern were obtained for each glass sample using X-ray diffractometer (PW 1700; Philips, Eindhoven, The Netherlands) to confirm the amorphous nature of prepared glasses and the existence of FAp layer on the surface of glass samples after *in vitro* studies [24]. The scanning electron microscope (SEM) (model 514A; Zeiss, Oberkochen, Germany) was employed to analyse the surface morphology of all glass samples before and after 21 days of *in vitro* studies in SBF solution. Fourier Transform Infra-Red spectra (FTIR) were recorded to find the functional groups in all the glass samples before and after *in vitro* studies using FTIR spectrometer (model 8700; Shimatzu, Tokyo, Japan) [25].

2.3. Cytotoxicity study

The Human osteoblast-like cell line (MG-63; National Centre for Cell Science, Pune, India) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) to analyse the primary toxicity of all the prepared glass samples. Cultured cells were maintained at 310 K in a humified 5% CO_2 incubator until the cells reached confluence. Cells were passaged and culture medium was changed twice in a week to avoid overgrowth.

Cells were counted using hemocytometer and diluted to a concentration of 3×10^4 cm^{-2} and seeded to the 96 wells (100 µl/well). 2.5 mg of each glass samples was soaked in a dish which contains 500 µl of DMEM for 24 h. The supernatant referred as conditioned medium which was added to the wells containing cells at different concentrations of 1, 10, 50 µl. Incubation was carried out for 24 h in CO_2 incubator. The media were removed from the wells and 500 µl of MTT assay (0.05%) was added to each wells and incubated for another 4 h at 310 K which converts MTT into purple formazan. Dimethyl sulfoxide (DMSO) was used to solubilize the formazan crystals in all the wells containing samples. The medium without samples served as control was also treated in the same manner. The optical density (OD) was determined for all the samples using spectrophotometer (ULTRA SPEC 2100 pro, Amersham Life Sciences, USA) at the wavelength of 570 nm. The obtained OD values were statistically analysed and the results were