Chapter 3 Materials and Experimental design

3.1 Preparation of bioactive glasses

The chemicals were used in this experiment are analytical reagent grade (all from Loba Chemie, Mumbai, India) such as quartz, sodium carbonate, calcium carbonate, barium carbonate, strontium carbonate, magnesium carbonate and ammonium dihydrogen orthophosphate as a source of SiO_2, Na_2O, CaO, BaO, SrO, MgO and P_2O_5 , respectively with a purity of 98- 99.9%. All were introduced in the form of their respective anhydrous state. The weighed batches were mixed for 30 minutes in an agate mortar and pestle and melted in a platinum crucible at 1400°C for 2 h in an electrical furnace. In order to ensure homogeneity, the glass melts were taken out of the furnace, poured on a preheated aluminum plate, cooled, crushed and re-melted in the furnace for an another period of 2 h. The bulk glass samples were annealed in a pre-heated furnace at 450 °C and after 1 h of annealing, the furnace was cooled to room temperature. The bulk glass samples were cut and polished into required dimensions. The polished glass samples were ultrasonically cleaned in an acetone bath.

3.2 Preparation of SBF

In order to carry out *in vitro* studies, I prepared simulated body fluid according to Kokubo [30] and that has inorganic ion concentrations similar to those of human body fluid in order to reproduce formation of apatite on bioactive materials in vitro as shown in **Table 3.1**. The SBF solution was prepared at 37 °C by dissolving reagent grade in the order given in **Table 3.2** NaCl, KCl, $NaHCO_3$, $MgCl_2.6H_2O$, $CaCl_2$ and KH_2PO_4 into double distilled water and it was buffered at pH=7.4 with TRIS (trishydroxy methyl aminomethane) and 1N HCl.

Table 3.1 Ion Concentrations in Simulated Body Fluid (SBF) and Human Blood Plasma

Ion	Concentration in SBF (mM/L)	Human Plasma (mM/L)
Na^{+}	142.0	142.0
K^{+}	5.0	5.0
Mg^{2+}	1.5	1.5
Ca^{2+}	2.5	2.5
Cl^{-}	147.8	103
HCO_3^{-}	4.2	27
HPO_4^{2-}	1.0	1.0
SO_4^{2-}	0.5	0.5

Table 3.2 Reagents for used for preparation of SBF

Sl. No.	Reagents	Amount
1.	NaCl	7.996 g
2.	$NaHCO_3$	0.350 g
3.	KCl	0.224 g
4.	$K_2HPO_4.3H_2O$	0.228 g
5.	$MgCl_2.6H_2O$	0.305 g
6.	1N HCl	40 ml
7.	$CaCl_2$	0.278 g
8.	Na_2SO_4	0.071 g
9.	$(CH_2OH)_3CNH_2$	6.057 g
10.	1N HCL	Appropriate amount for adjusting pH

3.3 Thermal behavior (DTA/TGA)

In order to identify the thermal behavior of the barium substituted bioactive glasses, the differential thermal analysis (SETARAM Instrumentation, France) was carried out on powdered samples in air up to 1000 ^{o}C using powdered alumina as a reference material with the heating rate of 10 ^{o}C min^{-1}. The glass nucleation and crystallization temperatures were obtained from the DTA results which were used for proper heat treatment for converting glass to their corresponding glass–ceramic.

3.4 Heat-treatment process for converting glass to glass-ceramics

Sometimes the bioactive glasses are used as coating materials on metal implants and composites therefore they are subjected to heat-treatment. Hence it is better to know their crystalline phases present. The prepared bioactive glass samples were heat-treated in two-step system, firstly nucleation temperature for the formation of nuclei sites and after holding for the specific time, it was then further heated to reach the second selected crystal growth temperature after holding for the specific time. The samples were left to cool inside the muffle furnace to room temperature at a cooling rate of 10 ^{o}C per min.

3.5 pH measurement

The stages of formation of hydroxyl carbonate apatite layer on the surface of the samples were checked by pH behavior of the SBF solution containing bioactive glasses. Each powdered bioactive glass samples were soaked in SBF (20 mg/ml) solution at 37 ^{o}C for different time periods and the pH of the leached solution was measured continuously using (Universal Bio-microprocessor, India) pH meter at room temperature. The pH values were recorded timely after definite intervals of time. The instrument was calibrated each time with standard buffer solutions of pH 4.0 and 7.0

3.6 Powder X-ray diffraction analysis

In order to identity the crystalline phase present in the bioactive glasses, glass-ceramics and SBF treated glass samples were ground to 75 microns and the fine powders were subjected to X-ray diffraction analysis (XRD) using RIGAKU-Miniflex II diffractometer adopted Cu-Kα radiation (λ = 1.5405A°) with a tube voltage of 40 kV and current of 35mA in a 2θ range between $10^{o}$and 80°. During measurement the step size and speed was set to 0.02◦ and 1° per min, respectively and was followed in the present investigation. The JCPDS-International Centre for diffraction Data Cards were used as a reference

3.7 FTIR spectrometric analysis

The functional groups of bioactive glasses containing BaO were investigated at room in the frequency range of 4000–400 cm^{-1} using a Fourier transform infrared (FTIR) spectrometer (VARIAN scimitar 1000, USA) in transmittance mode. The fine bioactive glass powdered samples were mixed with spectroscopic grade KBr in the ratio of 1 part of sample with 99 parts of KBr. The mixtures were subjected to an evocable die at load of 10 MPa to produce clear homogeneous discs. The discs were immediately put in the instrument for FTIR spectral transmission measurements and the spectra of samples were recorded. Further, the FTIR spectrometric analysis of the powdered bioactive glass samples containing SrO, MgO and Ag_2O was carried out in the frequency range of 4000–400 cm^{-1} in absorption mode using FTIR spectrometer Tensor 27 instrument (Bruker Optic, Germany).

3.8 In vitro bioactivity study of bioactive glass

The bioactivity of the prepared bioactive glass samples were examined through *in vitro* test. The test was performed by immersing samples in SBF solution (20

mg/ml) contained in a small polyurethane container and incubated at 37 ^{o}C in a static condition for time periods of 1, 3, 7, 14 and 30 days. After soaking, the samples were filtered, rinsed with double distilled water and dried in an electric air oven at 100 °C for 1 h. The formation of hydroxy carbonate apatite layer (HCA) on the surface of the bioactive glass samples were determined using FTIR, XRD and SEM & EDS techniques.

3.9 SEM and EDS analysis

The surface morphology of samples was analyzed before and after SBF treatment using a scanning electron microscope (SEM) (Inspect S50, FEI). The bioactive glass samples were cut into required dimensions and immersed into SBF for 14 days at 37 ^{o}C. Further, the samples were removed, washed with double distilled water and dried at 100 ^{o}C for 1 h and they were coated with gold by sputter coating instrument before their examination with SEM. I have also used (Inspect S50, FEI) as well as energy dispersive spectroscopy (EDS) (Oxford Instrument, X-act, Germany) for elemental analysis and mapping.

3.10 Density and flexural strength of glasses

The densities of annealed bioactive glass samples were determined by Archimede's principle with water as the immersion liquid. The flexural strength was measured using three point bending test. The bioactive glass samples were prepared into rectangular shape and they were ground and polished for required dimensions (60 mm length x 10 mm width x 5 mm thickness) using grinding and polishing machine. For flexural strength, the rectangular samples were subjected to three-point bending test according to ASTM C158-02 (2012). The test was performed at room temperature using Universal Testing Machine (AGS 10kND, SHIMADZU, Japan) having

cross-head speed at 0.5 mm/min and flexural strength was calculated using equation (3.1) and reported the mean and standard deviation.

$$\text{Flexural strength} = \frac{3PL}{2BD^2} \qquad --- (3.1)$$

Where, P is breaking load (Newton), L is length (mm), B is breadth (mm) and D is the thickness (mm) of the sample.

3.11 Compressive strength

The bioactive glass samples were subjected to compression load and the test was performed using Universal Testing Machine (Shimadzu, Japan) at room temperature (cross speed of 0.5 mm/min). Five samples were tested and the mean value and standard deviation was calculated. The compressive strength was calculated using the following equation (3.2):

$$\text{Compressive strength } (\sigma_c) = \frac{F}{A} \qquad -- (3.2)$$

Where F is the maximum compressive load during the test (N), A is the area of the specimen (mm^2).

3.12 Elastic modulus measurement by ultrasonic technique

The Young's, shear and bulk moduli of the polished bulk glass samples were determined by ultrasonic measurement gauge (45MG, Olympus, USA)[78]. Briefly, the ultrasonic wave velocities were recorded as longitudinal (VL) and transverse wave (VT). The velocities of sound wave propagated in the polished bioactive glass samples were measured using ultrasonic pulse-echo technique. The test was performed using two transducers as one was V112 for longitudinal wave (10 MHz) and another was V156 for the transverse wave (5MHz). The elastic properties such as Young's modulus

(E), shear modulus (S) and bulk modulus (K) were calculated. Five samples from each group were measured and the mean and standard deviation were calculated.

$$\text{Young's Modulus (E)} = \frac{{V_L}^2\, \rho\, (1+\sigma)(1-2\sigma)}{1-\sigma} \quad --- (3.3)$$

$$\text{Shear Modulus (G)} = {V_T}^2\, \rho \quad --- (3.4)$$

$$\text{Bulk Modulus (K)} = \frac{E}{3(1-2\sigma)} \quad --- (3.5)$$

3.13 *In vitro* cell culture studies

3.13.1 Cell lines and cell culture

Human osteosarcoma cell U2-OS was purchased from American Type Culture Collection (ATCC), Manahass, USA. The cells were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA), henceforth, called as complete medium. The cell line used in the study was free from mycoplasma.

3.13.2 In-vitro Cell viability assay

The cell viability of osteosarcoma cells was evaluated by a colorimetric XTT (sodium 3-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate) assay (Roche Molecular Biochemicals, Indianapolis, IN). Tumor cells were plated (5×10^3 cells/well) in a 96-well plate and exposed to bioactive glass samples and incubated at 37°C and 5% CO_2 for 18h. Optical Density was measured at 450 nm using Synergy HT Multi-Mode Micro plate Reader BioTek, USA [79]. The data was presented as the percentage of viable cell calculated from the following equation (3.6):

$$\% \text{ Cell Viability} = \frac{\text{Experimental } OD_{450}}{\text{Control } OD_{450}} \times 100 \quad \text{-------------- (3.6)}$$

3.13.3 In-vitro Cytotoxicity assay

The lytic activity of bioactive glasses against U2-OS cells was analyzed by non-radioactive cytotoxicity assay using the CytoTox 96 Non-Radioactive Cytotoxicity assay kit from Promega, USA [80]. Target cells (5×10^3) were added to 96-well tissue culture plate and exposed to bioactive glass samples and incubated for 18 h at 37^oC, 5% CO_2. Percent-specific lysis was determined using the following formula as given in equation (3.7):

$$\% \text{ Cytotoxicity} = \frac{(\text{Experimental - Effector Spontaneous - Target Spontaneous})}{(\text{Target Maximum - Target Spontaneous})} \times 100$$
-- (3.7)

3.13.4 Cell proliferation assay

Growth inhibitory potential of strontium contained bioactive glass samples against the tumor cells were studied by MTT assay. In a 96-well tissue culture plate, 5×10^3 cells/well were added and exposed to samples. Plates were incubated at 37^oC and 5% CO_2 for 48h. The cell proliferation was measured by Cell Titer 96 Non-Radioactive Cell Proliferation Assay (MTT) kit from Promega, USA. The data was presented as the percentage of inhibition of tumor cells and it was calculated from the following equation (3.8):

$$\% \text{ Growth Inhibition} = [1 - \frac{\text{Experimental } OD_{570}}{\text{Target } OD_{570}}] \times 100 \quad \text{--------- (3.8)}$$

Experimental optical density (OD) value is the reading of tumor cells exposed to Sr-contained glass samples and the Target OD value is the value of tumor cells only, cultured in absence of the Sr-glass samples [81].

3.13.5 Detection of apoptosis

Apoptotic cell death of tumor cells by bioactive glass samples was assessed by binding FITC-conjugated Annexin V. After 18 h of incubation, apoptotic cells were analyzed by staining with FITC-conjugated Annexin V and propidium iodide (PI) for 20 minutes in ice-cold PBS. Cells were washed in Annexin buffer and mounted on microscope slides with a drop of mounting medium to reduce fluorescence photo bleaching. The FITC-conjugated Annexin V positive cells were visualized under a fluorescence microscope (Nikon Eclipse 80i, Nikon, Japan).

3.13.6 Cell attachment

The bioactive glasses were cut into thin discs using diamond blade cutter and autoclaved (sterilisation) before the test. The U2OS cells (5×10^4 cells/well) were seeded on the surface of the discs. After 120 h incubation at 37°C, 5% CO_2 period the glass discs were taken out and rinsed three times with phosphate-buffered saline (PBS). The discs were dried at 37 °C for 24 h and a thin layer of gold was sputter-coated prior to examination under scanning electron microscopy (SEM). The morphology and cell attachment on the surface of the bioactive glass were observed using SEM (Zeiss, EVO 18, Germany) and energy dispersive spectroscopy (EDS) (Oxford Instrument, X-act, Germany) for elemental analysis.

3.14 Human blood compatibility

3.14.1 Hemolysis assay

The bioactive glasses were incubated with the blood sample. Hemolysis assay was performed according to the standard protocol [82]. In brief, an aliquot of each blood sample was centrifuged at 600 g for 5 minutes. 25 μl plasma aliquot was diluted with 225 μl Drabkin's reagent (Sigma) in a 96-well plate and mixed for 2 minutes under lateral agitation (300 rpm). After 10 minutes equilibration at room temperature, optical density was recorded at 540 nm in Synergy HT Multi-Mode Micro plate Reader BioTek, USA. Blood hemoglobin was determined by measuring the absorbance of 100-fold dilution of the whole blood in Drabkin's reagent at 540 nm. Saponin (2mg/ml final blood concentration) and PBS were used as positive and negative control, respectively. A sample of plasma without additives was considered as basal conditions. Standard calibration curve was obtained with the solutions containing 0.07 to 3.8 mg/ml bovine hemoglobin (Sigma) treated with Drabkin's reagent. The results were presented as percent hemolysis indicating the free plasma hemoglobin (mg/ml) and was measured as released hemoglobin divided by the total blood hemoglobin (mg/ml) multiplied by 100. All measurements were performed in triplicate.

3.14.2 Blood PBMC viability assay

Peripheral blood mononuclear cells (PBMC) were separated from whole blood following Ficoll-Hypaque density gradient centrifugation. The cells were washed in complete medium before use for the assay. The viability of PBMC cells was evaluated by a colorimetric XTT (sodium 3-[1-(phenylaminocarbonyl) -3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) assay (Roche Molecular Biochemicals, Indianapolis, IN). PBMC cells were plated (5 × 103 cells/well) in a 96-

well plate and exposed to bioactive glasses and incubated at 37 °C and 5% CO_2 for 18 h. Optical density was measured at 450 nm using Synergy HT Multi-Mode Micro plate Reader BioTek, USA. The data was presented as the percentage of viable cell calculated from the following equation (3.9).

$$\% \text{ Cell Viability} = \frac{\text{Experimental } OD_{450}}{\text{Control } OD_{450}} \times 100 \qquad \text{----------- (3.9)}$$

3.15 Statistical analysis

In this study, n reflects the number of times the experiments were performed independently in triplicate. The mean ± SD were calculated for each experimental group (n=3-5). Differences between groups were analyzed by unpaired Student's t-test and one- or two-way ANOVA analysis of variance depending on the requirement. One or two-way ANOVA followed by Holm-Sidak post-hoc multiple comparison tests was used to conduct pair wise comparisons using PRISM statistical analysis software (Graph Pad Software, Inc., San Diego, CA, USA). Significant differences among groups were calculated at $P<0.05$ or less (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ in control versus experimental group).