Chapter 2

MATERIAL AND METHODS

2.1 Selection of composition

The bioactive glass composition was formulated from 45S5 bioglasssystem. The 45S5 bioglass, having wt% composition [$45SiO_2$-$24.5Na_2O$-$24.5CaO$-$6P_2O_5$] was prepared. Then the proposed bioactive glass containing chemical composition (45–X) SiO_2-$24.5Na_2O$-$24.5CaO$-$6P_2O_5$-X (CeO_2, La_2O_3, Sm_2O_3 or Al_2O_3) (where X= 0-2 of REEs (CeO_2, La_2O_3, Sm_2O_3 or Al_2O_3) in wt %) was prepared. In the present study the weight percent of CaO (Merck, India), Na_2O (Merck, India) and P_2O_5 (Merck, India), was kept constant and SiO_2 (Merck, India) was partially replaced with CeO_2, La_2O_3, Sm_2O_3 or Al_2O_3.

2.2 Bioactive glass preparation

The compositions of bioactive glass as given in Table 2.1, were prepared by substitution of samarium oxide (0-4 wt %), cerium and lanthanum (0-2 wt %) and lanthanum and aluminum (III) oxide (0-2 wt %) in place of SiO_2 using the normal melting and annealing technique. Cerium and lanthanum oxide have been substitute to the glass composition for quartz in different concentrations to yield a non-charge balanced series of bioactive glass based on its dual role in the glass. The other components of the bioactive glass kept constant. Materials used include fine-grained quartz (Merck, India) for silica. Lime and soda were introduced in the form of their respective anhydrous carbonates (Merck, India). P_2O_5 was introduced in the form of ammonium dihydrogen orthophosphate [$NH_4H_2PO_4$] (Merck, India). All the materials were of analytical grade chemicals and used

without further purification. The weighed batches were mixed using planetary ball milling, agate mortar and pestle thoroughly for 40 minutes and melted in alumina and platinum crucibles of 100 ml capacity. The melting was carried out in an electric furnace at 1400±5°C for 3 hours in the air as furnace atmosphere and homogenized melts were poured on the preheated aluminum sheet. The prepared bioactive glass samples were directly transferred to a regulated muffle furnace at 470°C for annealing. After 2 hours of annealing, the muffle furnace was cooled to room temperature with a controlled rate of cooling at 20°C/ hr.

Table 2.1: ***Composition of REEs (CeO_2, La_2O_3, and Sm_2O_3) substituted bioactive glasses***

Sample Id	Weight %					
Batch 1.	**SiO_2**	**Na_2O**	**CaO**	**P_2O_5**	**CeO_2**	**La_2O_3**
BG	45	24.5	24.5	6	0.0	0.0
CeLa1	44	24.5	24.5	6	0.5	0.5
CeLa2	43	24.5	24.5	6	1.0	1.0
CeLa3	42	24.5	24.5	6	1.5	1.5
CeLa4	41	24.5	24.5	6	2.0	2.0
Batch 2.	**SiO_2**	**Na_2O**	**CaO**	**P_2O_5**	**CeO_2**	**La_2O_3**
Ce1	44.5	24.5	24.5	6	0.5	0.0
Ce2	44.0	24.5	24.5	6	1.0	0.0
La1	44.5	24.5	24.5	6	0.0	0.5
La2	44.0	24.5	24.5	6	0.0	1.0
Batch 3.	**SiO_2**	**Na_2O**	**CaO**	**P_2O_5**	**Sm_2O_3**	
BG	45	24.5	24.5	6	0.0	
Sm1	44	24.5	24.5	6	1.0	
Sm2	43	24.5	24.5	6	2.0	
Sm3	42	24.5	24.5	6	3.0	
Sm4	41	24.5	24.5	6	4.0	
Batch 4.	**SiO_2**	**Na_2O**	**CaO**	**P_2O_5**	**Al_2O_3**	**La_2O_3**
Alla-1	44	24.5	24.5	6	0.5	0.5
Alla-2	43	24.5	24.5	6	1.0	1.0
Alla-3	42	24.5	24.5	6	1.5	1.5
Alla-4	41	24.5	24.5	6	2.0	2.0

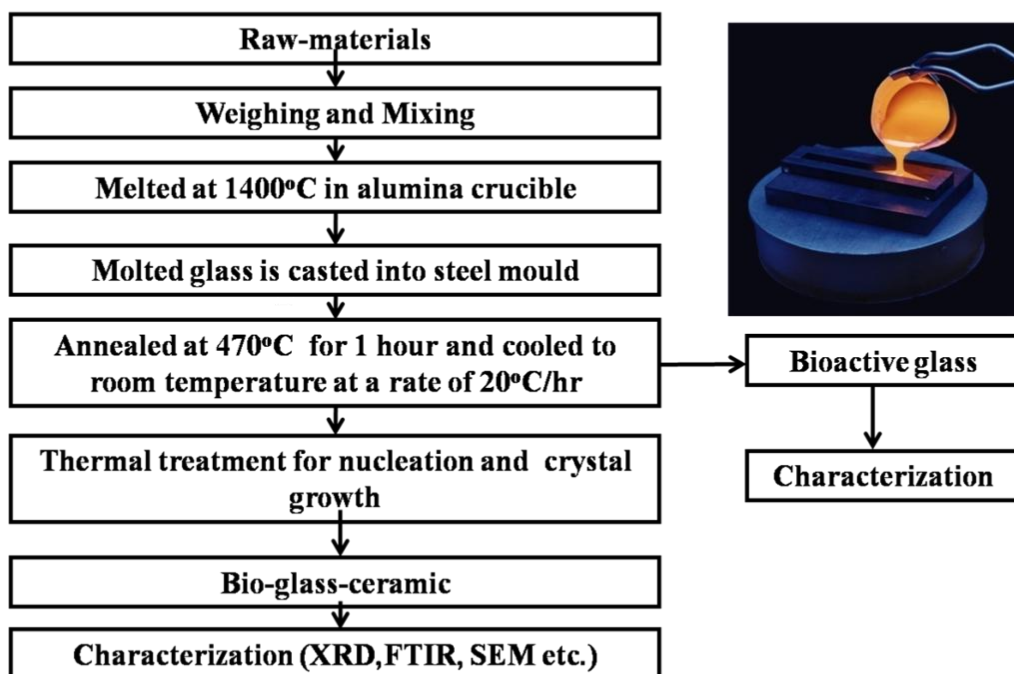

Figure 2.1: Flow chart - Preparation of Bioactive glass & their ceramic derivative

2.3 Differential thermal analysis (DTA)

To find out the nucleation and crystallization temperatures of the base bioactive glass cobalt and nickel oxide doped bioactive glasses, differential thermal analysis (DTA) were carried out. Fine powder of bioactive glass samples were made using an agate mortar and pestle and analyzed using a differential thermal analyzer (SETARAM, France) at a heating rate of 10°C per minute under a stream of argon atmosphere using alumina as a reference material. It was carried in the range of temperature 100 °C to 850 °C.

Figure 2.2: DTA/TGA Instrument [SETARAM, France]

2.4 Heat-treatment for conversion to glass–ceramic

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 as usual holding for the specific time. The samples were left to cool at room temperature inside the muffle furnace at a cooling rate of 10°C per hr. The temperatures were given in Table 2.2 for each bioactive glass system.

Table 2.2: ***Heat treatment temperatures used for nucleation and crystal growth of substituted bioactive glasses***

Sample	Nucleation (^{0}C)	Time (hrs)	Crystallization (^{0}C)	Time (hrs)
BG	602	6	764	3
CeLa1	601	6	630	3
CeLa2	600	6	614	3
CeLa3	599	6	606	3
CeLa4	598	6	609	3
Al_2O_3- La_2O_3substitutedbioactive glasses				
AlLa-1	601	6	630	3
AlLa-2	600	6	614	3
AlLa-3	599	6	606	3
AlLa-4	598	6	609	3

2.5 Preparation of SBF

Kokubo and his colleagues developed simulated body fluid that has inorganic ion concentrations similar to those of human body fluid in order to reproduce in vitro formation of apatite on bioactive materials [T. Kokubo et al. 2006]. The SBF solution was prepared by dissolving reagent-grade NaCl, KCl, $NaHCO_3$, $MgCl_2.6H_2O$, $CaCl_2$, Na_2SO_4 and $KH_2PO_4.3H_2O$ into double distilled water and it was buffered at pH=7.4 with TRIS (trishydroxymethyl amino methane) and 1N HCl at 37°C as compared to human blood plasma (WBC). The ion concentrations of SBF in mM/liter of solution are given in Table 2.3 [T. Kokubo et al. 2006].

Table 2.3*: Ion concentration (mM/litre) of simulated body fluid and human blood plasma*

Ion	**Na^+**	**K^+**	**Mg^{2+}**	**Ca^{2+}**	**HCO_3^-**	**HPO_4^{2-}**	**SO_4^{2-}**	**Cl^-**
Simulated body fluid	142.0	5.0	1.5	2.5	4.2	1.0	0.5	147.8
Human blood plasma	140.0	5.0	1.5	2.5	27.0	1.0	0.5	103.0

2.6 pH measurement

To measure pH of bioactive one gram glass powder was soaked in 10 ml of SBF solution at 37 °C for different time period and the pH was measured using Microprocessor based pH-EC meter (model-1611, ESICO-USA). The instrument was calibrated each time with standard buffer solutions of pH 4.00 and 7.00 at room temperature and pH values have been measured for different time periods at a fixed time interval.

Figure 2.3: Microprocessor based pH-EC meter (model-1611, ESICO-USA).

2.7 X-ray diffraction analysis of powders

In order to identity the crystalline phase present in the heat-treated bioactive glasses and bioactive glass-ceramics, the samples were ground to 75 microns and the fine powders were subjected to X-ray 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 20° and 80°. The step size and measuring speed was set to 0.02° and 1° min^{-1}, respectively was used in the present investigation. The JCPDS-International Centre for diffraction Data Cards were used as a reference.

Figure 2.4: X-ray diffractometer [RIGAKU-Miniflex II]

2.8 In Vitro Test by Fourier-transform infrared (FTIR) spectroscopy

The structure of glass & glass-ceramic were measured at room in the frequency range of 4000–400 cm^{-1} using a Fourier transform infrared spectrometer, (Bruker Tensor II FTIR, Germany). The fine glass & glass-ceramic powder samples were mixed with KBr in the ratio of 1:100 and the mixtures were subjected to an evocable die at load of 10 $tons/cm^2$ to produce clear homogeneous discs. The prepared discs were immediately subjected to FTIR spectrometer to measure the reflection spectra in order to avoid moisture attack. In order to investigate the formation of (calcium phosphate) apatite layer on the surface of the samples after immersion in SBF solution. 2.0 gram of the sample was immersed in 20 ml of SBF solution in a small plastic container at 37°C at the pH of 7.40 in an incubator at static condition for time periods 1, 3,7,15 and 30 days. After soaking, the samples were filtered, rinsed with doubly distilled water and dried in an air oven at 120 °C for 2 hours before FTIR spectrometric analysis.

Figure 2.5:FTIR Reflectance spectrometer [Bruker Tensor II FTIR, Germany]

2.9 Surface morphology by Scanning electron microscope (SEM)

2.0 gm of the powdered glass and glass-ceramic sample was soaked in 20 ml of SBF solution at 37 °C for different days and the pH was measured using microprocessor based pH-EC meter (model-1611, ESICO-USA). The instrument was calibrated each time with standard buffer solutions of pH 4.00 and 7.00 at room temperature and pH values were measured different time periods at a fixed interval. The glass powders (2 g) were pressed (load of 10 MPa) into pellet form using an evocable die to produce discs of 10 mm in dia for SEM analysis of bioactive glass samples. The pellets were immersed in SBF (20 ml) for 15 days at 37°C and the surface morphology of samples was analyzed before and after SBF treatment using a scanning electron microscope (SEM - Inspect S50, FEI). The samples were coated with gold (Au) by sputter coating instrument before analyzing by SEM.

Figure 2.6: Morphological image by Scanninc Electron Microscope [ZEISS EVO 18 SEM].

2.10 Mechanical Properties and Density Measurements

The density of the bioglass® samples was determined by Archimedes's principle (ASTM C20-00) where distilled water as an immersion fluid. The density was examined at 25˚C. Weight measurements have been carried out using electronic balance (Wensar High Precision Balance, HPB 220) with ± 0.0001 g accuracy. Density (ρ) of the sample was carried out by relation (1) as given below:

$$\text{Density } (\rho) = \frac{W_a}{W_a - W_b}\rho_b \quad \text{------------- (1)}$$

Where W_a and W_b is the weight of the sample in air and fluid respectively, and ρ_b is the density of fluid.

To measure the microhardness, bioactive glasses of cubic samples being well polished after that hardness performed by Digital hardness tester, the size of the cubic sample were 6mm x 6mm x 6mm according to ASTM Standard: C730-98. The load applied for indentations was ranging from 30 mN and 2000 mN, at the rate of 1 mm/s and permitted to equilibrate for 16 seconds prior to measurement. Microhardness (H) were evaluated using the relation (2) given below,

$$H = 1.854 (P/d^2) \qquad \text{-------------} (2)$$

Where P is the applied load on sample and d (m) is the diagonal of the impression

The molten glass was cast into a preheated rectangular mold and fabricated glass samples were fine ground and polished for 25×10×10 mm size. Now the samples were ready for 3-point bending test. The measurement was performed at 25 °C using Instron UTM ((H10KL, Tinius Olsen, USA) of 0.5 mm/min cross-head speed at full-scale load of 10kN. Flexural strength (σ) was evaluated according to ASTM C1674-11 shown in relation (3) given below,

$$\sigma = (3PL)/ (2bh^2) \qquad \text{------------} (3)$$

Where P is the applied load for the specimen being fractured, L, b and h is the length, width and height of the specimen respectively.

Figure 2.7: Universal Testing machine (TiniusOlsen H10KL)

2.11 Elastic Properties of glass and glass-ceramics

The ultrasonic wave velocities (longitudinal and shear) for cobalt oxide doped bioactive glass and base glass and its ceramic derivates were measured using the Olympus instrument (M-45, USA) made by USA. Bioactive glass and glass-ceramic samples were cut and polished in cubic pieces and the couplant glycerin was used for finding longitudinal velocities and sonfech shear gel for the shear velocities of bioactive glass and its ceramic derivative. Using the formula the Young's Modulus of Elasticity, Shear Modulus of Elasticity, bulk modulus of Elasticity and Poisson's Ratio were found.

Poisson's Ratio$=\frac{[1-2\left(\frac{V_L}{V_T}\right)^2]}{[2-2\left(\frac{V_L}{V_T}\right)^2]}$

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

Shear Modulus (S) $= V_T^2\rho$

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

Where ρ is the density and σ is the Poisson's ratio the samples

Figure 2.8: Olympus instrument (M-45, USA)

2.12 Assessment of biocompatibility

2.12.1 Cell Culture

The mouse fibroblast, L929 cell line was procured from the National Centre for Cell Science, Pune, India, and cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics-mycotic solution. It was incubated under a humidified environment of 5% CO_2 and 95% air at 37 °C. The cells were sub-cultured at an interval of every 5th day and used up to 10th passage for the experiments.

2.12.2 Cellular compatibility assay

Cellular compatibility of bioglass® was evaluated by using colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. At about 70 to 80 % confluence cells were harvested and re-suspended into fresh culture media at a density of 10^5 cells/ml. 100 μl of cell suspension was plated in 96-well tissue culture plates and incubated overnight and then, old media were replaced with fresh culture media and exposed to different content of test samples in triplicate for 48 h. The stock concentrations of samples were 20 mg/ml suspended in 10 mM PBS, and then it was diluted into 10 times in culture medium and further serially diluted in the well using micropipette.

$$\%\text{Cell viability} = \frac{\text{Experimental } OD_{570}}{\text{Control } OD_{570}} \times 100$$

The control negative was taken the cells without any treatment and the positive control cells were treated with 10 % DMSO. After incubation, culture media were replaced with 50 μL of PBS containing MTT (final concentration: 0.5 mg/mL) in each well were again incubated for 4 h at 37 °C. Then, the supernatant was removed and 100 μL of DMSO were added to each well to solubilize the water-

insoluble violet formazan crystals and absorbance was deliberate in a microplate reader (Multiskan™ FC Microplate reader, USA) at 570 nm.

Figure2.9: Microplate reader [Multiskan™ FC Microplate reader, USA]

2.12.3 Cell proliferation

The cell proliferation assay were carried out using L929 cell lines cultured About 70 to 80 % confluence cells were harvested and re-suspended into fresh culture media at a density of 10^5 cells/ml. 100 µl of cell suspension were plated in 96-well tissue culture plates and incubated for 24 hours as above. Then old media were replaced with fresh culture media and cells were treated with samples at a concentration equivalent to the IC_{50} value of the glass sample for 1, 3 and 5 days in triplicate. Then, MTT assay was done on the cells which grown in the presence of the samples after incubation to compare the effect of a sample on the cellular growth.

$$\%\text{Growth inhibition} = [1 - \frac{\text{Experimental OD}_{570}}{\text{Control OD}_{570}}] \times 100$$

2.12.4 Blood compatibility by Hemolysis assay

Hemolysis assay is a basic test for biocompatibility of a sample and it was also assayed in this study for bioactive glass. It was taken place when red blood

cells (RBC) come in contact with toxic or unfavorable materials, and it is essential to confirm the bioactive materials before their clinical trials. Hence, the in vitro blood compatibility of bioactive glasses was evaluated for percentage hemolysis. The blood was collected from healthy non-alcoholic and non-medicated adult human male donor in an EDTA treated collecting tube. The RBCs were separated by centrifugation and dilution in normal saline and collected RBCs were re-suspended into 10mM PBS and corpuscles were calculated for their density. 10^7 RBCs were taken in 1 ml of 10mM PBS and then a sheet of bioglass® having the dimension of 4 mm X 4 mm was released in each tube and incubated for the experiment. RBCs suspended into the double distilled water was considered as positive control with 100 % hemolysis and RBCs suspended 10mM PBS, considered as negative control in which no hemolysis occurred. All data were compared within these groups and percentage hemolysis was evaluated by,

$$\%\text{Hemolysis} = \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{negative control}}}{\text{OD}_{\text{positive control}} - \text{OD}_{\text{negative control}}} \times 100$$

2.12.5 Detection of cell apoptosis

The compatibility and safety of the glass were evaluated with detection of the cell death induced by the samples (if any) amongst the L929 cell lines by Ethidium bromide-Acridine orange double staining.

Figure 2.10: Fluorescence optical microscopy [Dewinter, India]

The growing cells in culture about 70 to 80 % confluence cells were harvested and seeded as above. Then, after 24 hours incubation, cells were exposed to the samples for further 24 hours and fluorescence microscopy was done to study the cellular and nuclear changes to the substituted samples.