## 2. Materials and methods:

### 2.1 Sample preparation

Glass samples were prepared in five different systems by taking the materials as reagent grade fine-grained quartz ($SiO_2$), calcium carbonate ($CaCO_3$), sodium carbonate ($Na_2CO_3$), ammonium dihydrogen phosphate ($(NH_4)H_2PO_4$), lithium carbonate ($Li_2CO_3$), potassium carbonate ($K_2CO_3$), strontium carbonate ($SrCO_3$), copper oxide (CuO), and a mixture of ($TiO_2$ + $ZrO_2$) in the required stoichiometric ratio in mol%. The required amounts of analytical reagent grade $Al_2O_3$ were added in the batch for the partial substitution of $Li_2O$, $K_2O$ and SrO in first three systems. In fourth system CuO was added in the batch for the partial substitution of CaO. In fifth system the required amounts of analytical reagent grade ($TiO_2$ + $ZrO_2$) were added in the batch for the partial substitution of silica. The raw materials for different samples were properly weighed. The mixing of different batches was done for 30 minutes and then after that, they were melted in a 100 ml platinum-2% rhodium crucible for 4 hr kept in a globar electric furnace at 1400°C in air atmosphere. The temperature of the furnace was controlled within ± 10°C by an automatic temperature indicator-cum-controller. Further, the glass melt was taken out of the furnace, poured on an aluminium sheet in a rectangular mould and transferred immediately to an annealing furnace. The glass samples were properly annealed at 500 °C for 1 h and cooled slowly to room temperature with a controlled rate of cooling inside the muffle furnace to remove the stress and strain from the glass. A part of the annealed bioactive glass samples was cut, ground and polished for measurement of its physical and mechanical properties. The other parts of the samples were crushed in a pestle mortar and then ground in an agate mortar to make fine powders for measurements of its bioactivity

and other properties with various experimental techniques such as XRD, FTIR spectrometry, SEM analysis and pH measurements.

**2.2 Density, compressive strength and ultrasonic wave velocity measurements:**

The densities of the samples were determined by Archimedes's principle with water as the immersion liquid. The measurements were carried out at room temperature. Compressive strength of bioactive glass samples was measured by universal testing machine. Young's Modulus, shear modulus and bulk modulus of glass samples were determined by ultrasonic measurement gauge. The ultrasonic wave velocities were recorded as longitudinal ($V_L$) and transverse wave ($V_T$). The velocities of sound wave propagated in the polished bioglass samples were measured using ultrasonic pulse-echo techniques (45MG, Olympus, USA). The test was performed using two transducers, one was V112 for longitudinal wave (10 MHz) and another was V156 for the transverse wave (5 MHz). Glycerin and burnt honey was used as bonding material between samples and transducers. Elastic properties such as Young's modulus (E), shear modulus (S) and bulk modulus (K) using the following standard equations:

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

$$\textbf{Shear Modulus}(\mathbf{G}) = \mathbf{V_T}^2\,\boldsymbol{\rho} - - - (\mathbf{2})$$

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

Where, ρ is density and σ is the Poisson's ratio of glass samples.

## 2.3 X-ray diffraction (XRD)

In order to identity the crystalline phase present in the bioactive glasses and SBF treated glass samples were ground to 75 μm and the fine powders were subjected to X-ray diffraction analysis (XRD) using RIGAKU-Miniflex II diffractometer adopted Cu-Kα radiation (λ = 1.5405 Å) with a tube voltage of 40 kV and current of 35 mA in a 2θ range between 20° and 80°. During measurement the step size and speed were set to 0.02° and 1° per min, respectively and were followed in the present investigation. The JCPDS-International Centre for diffraction Data Cards were used as a reference.

## 2.4 Fourier transform infrared (FTIR) spectrometry:

The in-vitro bioactivity of chemically treated samples in SBF solution were assessed by evaluating the formation of hydroxy calcium phosphate (HCA) layer on the surface of the samples after immersion in SBF solution by FTIR spectrometry. The infrared absorption spectra of the bioglasses before and after immersion in SBF were recorded at the room temperature in the spectral range 4000-400 $cm^{-1}$ using an FTIR reflectance spectrometer (TENSOR 27, Bruker, Germany).

## 2.5 Scanning electron microscope (SEM):

The surfaces of bioactive glasses were analyzed before and after immersion in SBF solution by scanning electron microscopy. The samples were coated with plasma gold plate before SEM analysis. A scanning electron microscope (Inspect 50 FEI) was used to determine the surface microstructure of bioactive glass samples.

## 2.6 In vitro bioactivity analysis:

In order to identify the HCA layer formation, the glass samples were immersed in SBF solution at 37.4°C for different time periods from 1 to 28 days. The simulated body fluid

(SBF) solution was prepared according to the formula described by Kokubo et al (Kokubo et al., 1990). Table 2.1 shows the reagents for the preparation of SBF. The pH of the SBF solution was measured using a digital pH meter after immersion of samples for different time periods.

**Table 2.1 Reagents for preparation of SBF.**

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

### 2.7 Cell lines and cell culture

Human osteosarcoma cell U2-OS was purchased from American Type Culture Collection (ATCC), Manassas, VA, 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. Peripheral blood mononuclear cells (PBMC) were isolated from freshly collected blood by Ficoll-Hypaque density gradient. The mononuclear cells were collected, washed in complete medium before use for the assay.

**2.8 Cell viability assay**

Effect of bioactive glass samples on the viability of U2-OS tumor 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 ($5\times10^3$ cells/well) in a 96-well plate and exposed to single concentration (25 mg/ml) of different bioactive glass samples and incubated at 37°C, 5% $CO_2$, for 18h. For human PBMC, the cells were incubated with different concentrations of bioactive glass samples and incubated at 37°C, 5% $CO_2$, for 18h. Optical density (OD) was measured at 450 nm using Synergy HT Multi-Mode Micro plate Reader BioTek, USA (Hira *et al*., 2014). The data was presented as the percentage of viable cell calculated from the following formula:

$$\% \text{ Cell Viability} = \frac{\text{Experimental } OD_{450}}{\text{Control } OD_{450}} \times 100$$

**2.9 Cytotoxicity assay**

The lytic activity of bioactive glass samples against U2-OS cells was measured by non-radioactive cytotoxicity assay using the CytoTox 96 Non-Radioactive Cytotoxicity assay kit from Promega, USA (Manna *et al*., 2013). Target cells ($5\times10^3$) were added to 96-well

tissue culture plate and exposed to serial concentrations of samples and incubated for 18h at 37°C, 5% $CO_2$. Percent-specific lysis was determined using the following formula:

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

**2.10 Tumor cell proliferation assay**

Growth inhibitory potential of bioactive glass samples against the tumor cells was studied by MTT assay (Hira *et al*., 2014). In a 96-well tissue culture plate, $5\times10^3$ cells/well were added and exposed to serial concentrations of bioactive glass samples. Plates were incubated at 37°C, 5% $CO_2$ for 48h. The cell proliferation was measured by CellTiter 96 Non-Radioactive Cell Proliferation Assay (MTT) kit from Promega, USA. The data presented as the percentage of inhibition of tumor cells and was calculated from the following formula:

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

Experimental OD (optical density) value is the reading of tumor cells exposed to various concentrations of bioactive glass samples and the Target OD value is the value of tumor cells only, cultured in absence of the bioactive glass samples.

**2.11 Hemolysis assay**

Hemolysis assay was performed according to the standard protocol with some modifications (Kuznetsova *et al*., 2012). For the time dependent kinetics, single concentration (20mg/ml) of different bioactive glass samples was incubated with the

blood sample. Aliquots of each blood sample were centrifuged at 600g 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 are 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.

**2.12 Detection of apoptosis**

Apoptotic cell death in U2-OS cells by bioactive glass samples (25mg/ml) was assessed by binding FITC-conjugated Annexin V (Hira *et al*., 2014). After 12h 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 pictures were taken 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).

## 2.13 Phagocytosis assay

Human macrophages were cultured from monocytes on glass coverslips in complete medium for 5-7 days at 37°C, 5% $CO_2$. Bioactive glass samples (25mg/ml) were added to the macrophage cultures and were allowed to phagocytose for 24 h. The cover slips were washed, dried, fixed with ethanol and stained with Giemsa stain. Phagocytosed ceramic materials were counted at room temperature using microscope (Nikon Eclipse 80i, Nikon, Japan). Phagocytosis analysis was performed by counting the phagocytic macrophages captured under the microscope. The percentages of phagocytic macrophages were calculated by dividing the number of phagocytic macrophages by total number of macrophages multiplied by 100.

## 2.14 Statistical analysis

In this study, n reflects the number of times experiments were performed independently in triplicate. The mean ± SD were calculated for each experimental group (n=3-4). 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 groups).

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