Chapter 3

Materials and methods

2.1 Materials

2.1.1 For glass-scaffold preparation

2.1.1.1 Melt-route

- Polyurethane (PU) foam [Analytical reagent (AR) grade]
- Quartz **(**SiO_2**)** (for 1393 glass scaffold)
- H_3BO_3 (for 1393B3 glass scaffold)
- Sodium carbonate (Na_2CO_3)
- Calcium carbonate ($CaCO_3$)
- Potassium carbonate (K_2CO_3)
- Magnesium oxide
- Ammonium dihydrogen orthophosphate
- Cupric oxide

(Loba chemie, Mumbai, India; Assay 98–100 %) and

- Binders (PVA).

2.1.1.2 Sol-gel route

i. TEOS (Assay 98%, Sigma-Aldrich, US); **ii.** TEP (Assay 98%, Sigma-Aldrich, US); **iii.** $Ca(NO)_3.4H_2O$; **iv.** $Mg(NO)_3$ **v.** $NaNO_3$ **vi.** KNO_3 and **vii.** $Zn(NO_3)_2$ (Assay >99%, Loba chemie, IN); **viii.** $Sr(NO_3)_2$ **ix.** Hydrolysis agent (i.e., HNO_3) and **x.** Deflocculants and Binders (PVA) and **xi.** Polyurethane (PU) foam.

2.1.2 For SBF (simulated body fluid) preparation

Kokubo et.al., postulated in vivo alike response of any synthetic biomaterials could be reproduced by apatite like layer formation by simulation of blood plasma. The SBF solution in accordance with Kokubo was prepared by dissolving required amount of NaCl, $MgCl_2 \cdot 6H_2O$ (Merck,Germany), KCl, $K_2HPO_4 \cdot 3H_2O$, $CaCl_2$, $NaHCO_3$, and Na_2SO_4 (Loba chemie, India) into milipore double distilled water, buffered at pH 7.40 with TRIS (tris-hydroxymethyl aminomethane) and 1 (M) HCl solution at 37℃.

2.1.3 Cells/ cell lines

Biological compatibilities of the scaffolds were assessed by using the following cell/ cell lines

- L929 (mouse fibroblast) cells from NCCS, Pune, IN
- U2OS (Human osteosarcoma) cell lines from, ATCC, Manassas, US
- NIH/3T3 (mouse normal embryonic) cells from NCCS, Pune, IN
- SCC-25 (human squamous carcinoma) cells from ATCC, Manassas, US
- HCT 116 (human colon epithelium) cell lines from ATCC, Manassas, US).
- mBMSCs **(**mouse bone marrow stromal cells) derived obtained from the bone marrow of 8 weeks old wild type CD1 mice
- PBMC (Peripheral blood mononuclear cells; Monocytes and Lymphocytes) obtained from mouse blood from tail vein

2.2 Methods

2.2.1 Bioactive glass-scaffold preparation

The compositions of bioactive 1393 and 1393B3 as tabulated below (*Table 2.1*) were prepared by melt-route or sol-gel route. The prepared glasses were then foam replicated to obtain desired scaffolds.

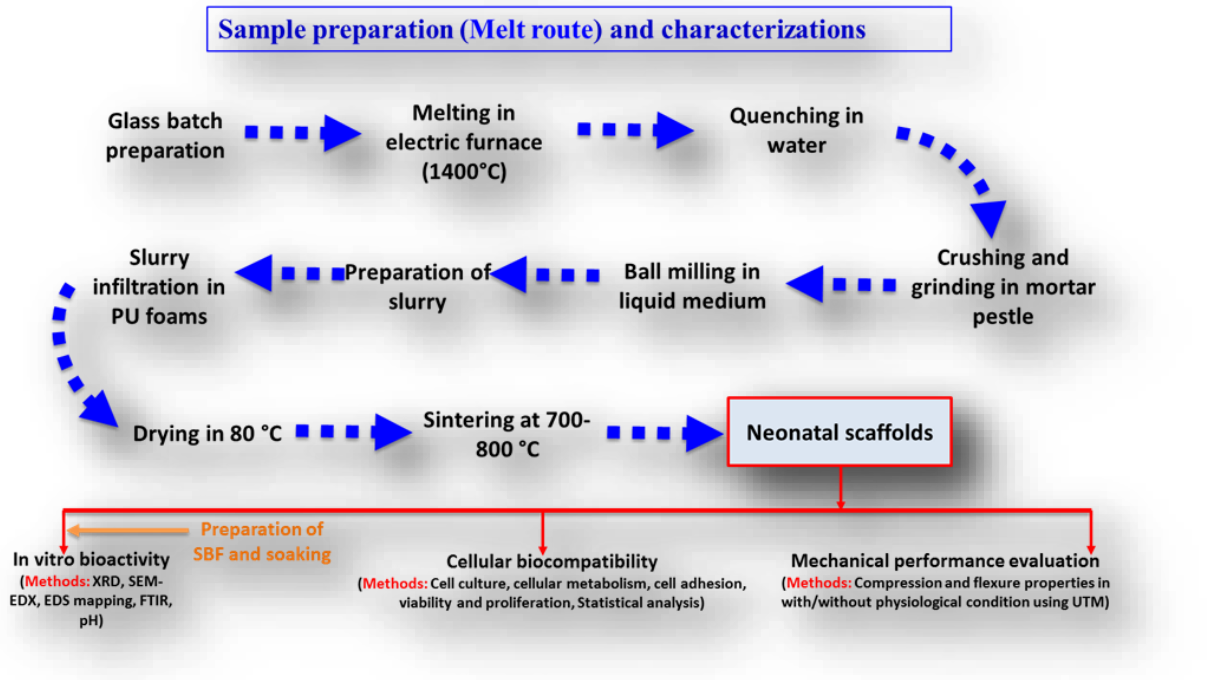
Table 3.1: Chemical composition of therapeutic ions substituted 1393/1393B3 glass (mol%)

CuO substitution for SiO_2 in 1393 glass							
Sample codes	SiO_2	CaO	Na_2O	K_2O	MgO	P_2O_5	CuO
1393	54.65	22	6	7.9	7.7	1.75	0
1393-1Cu	53.65	22	6	7.9	7.7	1.75	1
1393-2Cu	52.65	22	6	7.9	7.7	1.75	2
1393-3Cu	51.65	22	6	7.9	7.7	1.75	3
ZnO substitution for SiO_2 in 1393 glass							
Sample codes	SiO_2	CaO	Na_2O	K_2O	MgO	P_2O_5	ZnO
BG	54.65	22	6	7.9	7.7	1.75	0
Z1BG	53.65	22	6	7.9	7.7	1.75	1
Z2BG	52.65	22	6	7.9	7.7	1.75	2
Z3BG	50.65	22	6	7.9	7.7	1.75	4
SrO substitution for CaO in 1393 glass							
Sample codes	SiO_2	CaO	Na_2O	K_2O	MgO	P_2O_5	SrO
S1	54.65	22	6	7.9	7.7	1.75	0
S2	54.65	20.9	6	7.9	7.7	1.75	1.1
S3	54.65	17.6	6	7.9	7.7	1.75	4.4
S4	54.65	11	6	7.9	7.7	1.75	11
S5	54.65	0	6	7.9	7.7	1.75	22
CuO substitution for B_2O_3 in 1393B3 glass							
Sample codes	B_2O_3	CaO	Na_2O	K_2O	MgO	P_2O_5	CuO
BBG	54.6	22.1	6	7.9	7.7	1.7	0
C1BBG	53.6	22.1	6	7.9	7.7	1.7	0.5
C2BBG	52.6	22.1	6	7.9	7.7	1.7	1
C3BBG	50.6	22.1	6	7.9	7.7	1.7	2

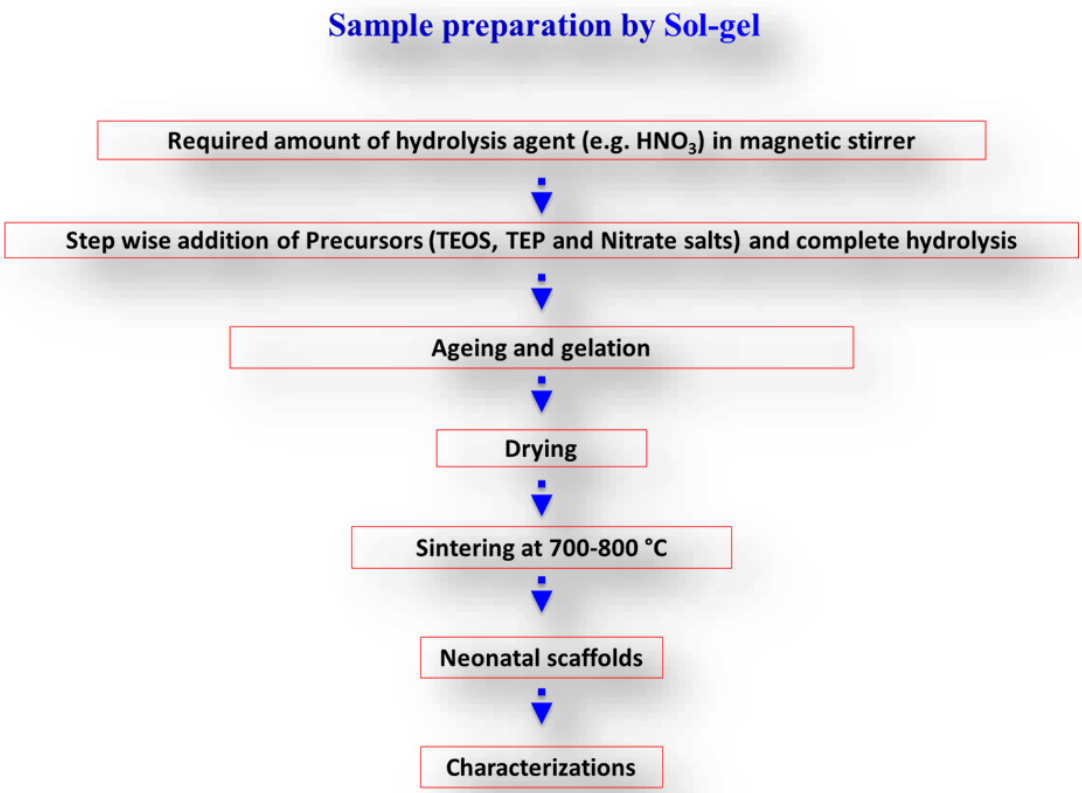
The CuO incorporated 1393 and 1393B3 glasses were prepared via melt-route using the general formula (54.6-X)SiO_2. 6Na_2O. 7.9 K_2O. 7.7 MgO. 22 CaO. 1.74 P_2O_5. XCuO (where X=0, 1, 2, 3; mol %), and (54.6-X)B_2O_3. 6Na_2O. 7.9 K_2O. 7.7 MgO. 22 CaO. 1.74 P_2O_5. XCuO (X=0, 0.5, 1.0, and 2.0; mol %) respectively. Whereas, the sol-gel glasses doped with ZnO and SrO were performed using the formulas (54.6-X)SiO_2-6Na_2O-7.9K_2O-7.7MgO-22CaO-1.74P_2O_5-XZnO and 54.6SiO_2-6Na_2O-7.9K_2O-7.7MgO-(22-X)CaO-1.74P_2O_5-XSrO (X=5%, 20%, 50%, and 100% of CaO; mole%).

In melt route, proportionate amount of AR grade anhydrous reagents were weighed and mixed for thoroughly before melting. The reagents used for preparations of therapeutic ions incorporated glass scaffolds were analytical reagent (AR) grade quartz, sodium carbonate, calcium carbonate, potassium carbonate, magnesium oxide, ammonium dihydrogen orthophosphate, cupric oxide from Loba chemie, India (Assay: 98–99.9%).

The batches after initial processing were melted in an electric furnace at 1400±100 ^{0}C in Platinum/ Alumina (high quality) crucibles. The glasses after being air quenched at room temperature, crushed, ground using pestle and agate mortar. The powdered samples after 4-12h additional ball-milling by zirconia/alumina balls, and using ethanol as grinding media and ethanol (Merck, Germany) slurries were prepared by mixing with Millipore double distilled water, ethyl alcohol' and binder cum deflocculant (e.g. PVA)Polyurethane packaging (PU) foams after cutting into required dimensions were slurry infiltrated by dipping them into the slurries. Porous foams were used as sacrificial template material aiming tailoring into trabecular bones' architecture. The excess slurry was cut out from the surfaces, followed by subsequent heat treatment. The heat treatment involves initial strengthening, removal of additives and sintering. Foam replicated neonatal scaffolds were prepared after the heat treatments.



The ZnO and SrO derived 1393 glass scaffolds were synthesized via wet-chemical sol-gel route through stepwise hydrolysis of precursors. Briefly, required amount of 1(M) HNO_3 was added with proportionate amount of Tetraethylsilane (assay 98%, Sigma-Aldrich, IN), TEP (assay 99%, Sigma-Aldrich, IN), $Mg(NO)_3$, $Ca(NO)_3.4H_2O$, KNO_3, $NaNO_3$, $Sr(NO_3)_2$, and$Zn(NO_3)_2$ (Assay >99%, Loba chemie, IN) one after another in sequence maintaining 45 min gap to allow complete hydrolysis of the precursors. The molar ratio of (H_2O+HNO_3)/ (TEOS+TEP) for a particular study set was maintained at a constant value. PU foams were dipped into the sols as soon as the thickening of the sols start. The resultant gel infiltrated foams were then aged, followed by heat treatment to prepare the foam replicated scaffolds.



2.2.2 In vitro bioactivity

- XRD: RIGAKU Miniflex II diffractometer, @ 3° min^{-1} over angular range (2θ) 10-80°, Ni filter and Cu-K_α source with λ = 1.5405 Å.
- pH: Universal pH meter (Universal Bio microprocessor, IN) at particular intervals (day1, day3, day5..)
- SEM: Morphological micrographs of the samples using Scanning Electron Microscope (SEM) EVO|18, ZEISS (US)
- EDX: Energy Dispersive X-ray spectroscopy equipped with SEM (EVO|18, ZEISS (US) for elemental quantification of the scaffolds surface.

- EDS mapping: Spatial distribution of elements over scaffolds in order to evaluate HAp nodules on scaffolds surface.

- FTIR: Fourier Transform Infrared Spectrometer (BRUKER Tensor 27, Germany) @within the frequency range of 4000- 400 cm^{-1} taking 32 scans with a resolution of 4 cm^{-1}.

2.2.3 Cell isolation & cell culture

- Mouse bone marrow derived stromal cells: from the bone marrow of 8 weeks old wild type CD1 from femur and tibia were flushed out with complete isolation media (CIM) in RPMI 1640, supplemented with 10% fbs (Himedia, India), 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen, Carlsbad, CA). Washed twice with PBS, filtered (70 µM nylon mesh).Cells were cultured in a 6 well cell culture plate in 5% CO_2 incubator at 37 °C. Iscove modified Dulbecco medium (IMDM; GIBCO) supplemented with 10% FBS, 5 ng/mL basic fibroblast growth factor (βFGF), 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA), and 12 µM L-glutamine (Invitrogen, Carlsbad, CA). BMSCs at 90% cell confluence were continued subcultured at 1:2split ratio in CCM supplemented with βFGF (5 ng/mL) for several passages

- PBMC (Peripheral blood mononuclear cells; Monocytes and Limphocytes): Mouse blood from tail vein was collected and isolated by gradient centrifugation using Ficoll-Hypaque. washed with RPMI-1640, supplemented with 5% FBS, re-suspended and collected by diluting with 10µl WBC diluting fluid and cultured with complete medium.

- L929, U2OS, NIH/3T3, SCC-25, and HCT 116 purchased and cultured in complete culture media supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA) in 5% CO_2 and 95% humidified atmosphere at 37 ˚C.

2.2.4 Biological compatibility of cells in the scaffolds

- Cell viability by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and XTT [sodium 3-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate] assay using cells (U2OS, NIH/3T3, mBMSC, PBMCs, L929 and SCC-25) in 6 and 96 well plates seeded onto scaffolds. OD was measured @475nm

$$\% \text{ Cell Viability} = \frac{\text{Experimental } OD_{475}}{\text{Control } OD_{475}} \times 100$$

- Cell proliferation Cells (U2OS, NIH/3T3, mBMSC, PBMCs, L929 and SCC-25) proliferation by CellTiter 96® Non-Radioactive Proliferation assay (MTT) kit from Promega using the formula

$$\% \text{ Proliferation} = \frac{\text{Experimental } OD_{570} - \text{Blank}}{\text{Control } OD_{570} - \text{Blank}} \times 100$$

- Cytotoxicity: The cell lysis was measured as LDH release after 16h of cells culture in Cytotox 96® Non-Radioactive cytotoxicity assay (MTT) kit from Promega. LDH released in the culture supernatant was measured with 30min coupled enzymatic assay

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

- Apoptosis: Cells (U2OS, NIH/3T3, mBMSC, PBMCs, L929 and SCC-25) were treated with 10µg/ml indicated materials for 48h, and were washed three times with PBS for 5 min. After fixing the cells with cold methanol for 20 min and another three PBS washes for 5 min were done and permeabilization was done using Triton-× for 5 min and then washed again with PBS. Viable (live;green) and apoptotic (dead; red/orange) cells were visualized after dual AO/EB staining in Leica DMi8 (Germany) at 400× magnification.

- Hemocompatibility: Hemolysis assay was prepared in accordance with the standard protocol i.e. an aliquot blood sample was centrifuged at 600×g for 10 min for each assay followed by dilution of 25 µl supernatant aliquot with 225 µl Drabkin's reagent (Sigma) in a 96 well plate and homogenization for 2 minutes under lateral agitation (300 rpm). OD was measured using Synergy HT Multi-Mode Micro plate Reader (BioTek, USA)

$$\% \, Hemolysis = \frac{OD540_{sample} - OD540_{negetive\ control}}{OD540_{positive\ control} - OD540_{negetive\ contol}} \times 100$$

- Cell adhesion: Cell adhesion study onto the glass scaffolds is one of the foremost techniques used to evaluate the cell-scaffolds interactions and biocompatibility

of the scaffolds. The visual inspection of cellular attachments onto the glass surface indicates biological compatibility of the scaffolds.

2.2.5 Osteogenesis: by ALP activity, and Osteogenic genes expression

- ALP activity Cells (mBMSCs) were grown with scaffolds, harvested by trypsinization. Cells lysates were prepared with lysis solution (2% Triton ×-100 and 5mM $MgCl_2$) and the lysate was subjected to the alkaline phosphatase activity assay using Alkaline Phosphatase Diethanolamine activity Kit from Sigma as per manufacturer's protocol (Cat. No. AP0100; Sigma)

$$ALP\ activity = \left(\frac{(Abs_{test} - Abs_{blank}) \times df \times Vf}{18.5VE}\right)_{\lambda=405nm}\ \text{IU/L}$$

Where, Df =dilution factor; Vf = volume of assay; VE = volume of test sample and 18.5 is the molar extinction coefficient at 405nm

- Osteogenicgenes expression by RT-qPCR Total RNA extraction was done using Total RNA Miniprep Purification Kit HiPurA™ (Himedia, IN). Isolated RNAs were then reversed transcribed to complementary DNA (cDNA) using HiScript One Step RT-PCR (reverse transcriptase polymerase chain reaction) Kit. The primer specific osteogenic genes osteopontin (OPN), osteocalcin (OCN) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using Prima-96™ Thermal Cycler after denaturation (at 95°C for 1 min 45 s in 26 cycles of a three step cycle i.e. 95°C for 15 s, 57°C for 30 s, and 72°C for 1 min 55 s), and extension (at 72°C for 10 min). The transcribed product was visualized with 1% agarose gel

electrophoresis and images of gel bands were analyzed with ImageJ software for quantification. The optical intensities were normalized with GAPDH

Table 3.2: *Osteopontin (OPN), Osteocalcin (OCN) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer for quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) analysis*

Genes	Sequence	Primer (Forward)	Primer (Reverse)
OPN	5'→3'	CTTTCACTCCAATCGTCCCTAC	GCTCTCTTTGGAATGCTCAAC
OCN	5'→3'	GGGAGACAACAGGGAGGAAAC	CAGGCTTCCTGCCAGTACCT
GAPDH	5'→3'	AGGAGTATATGCCCGACGTG	TCGTCCACATCCACACTGTT

2.2.6 Physico-mechanical properties of the scaffolds

- Strengths of the scaffolds were measured in terms of compression (σ_c), flexure (σ_f) and their elastic modulii (E_f) . Three point bend or flexural stress of the porous scaffolds with required dimensions was measured in accordance with ASTM C1674-11 using universal testing machine (UTM) H10KL (Tiniusolsen, US). The strengths were calculated as follows:

$$\text{Compressive stress, } \sigma_c = \frac{P}{A}$$

$$\text{Flexural stress, } \sigma_f = \frac{3PL}{2BD^2}$$

$$\text{Strain, } \varepsilon = \frac{6Dd}{L^2}$$

$$\text{Modulus of flexure, } E_f = \frac{L^3 m}{4BD^3}$$

Where P=load (Newton), A= surface area of load (mm^2), L =outer span length (mm), B= breadth of the scaffolds (mm), D= depth of the scaffolds (mm), d= deflection/ midspan (center of the beam) displacement (mm) and m= the gradient (i.e., slope) of the initial straight-line portion of the load deflection curve, (N/mm).

- Porosity: The apparent porosity (AP) of the bioactive scaffolds were determined by Archimedes' principle (ASTM C20-00) using liquid (water) as buoyant by the following formulas

$$\% \text{ AP} = \frac{w_b - w_a}{w_b - w_c} \times 100$$

- Chemical durability by weight loss: Weight loss techniques was used to measure the durability of a glass by the formula

$$\% \text{ Weight loss} = \frac{W_i - W_f}{W_i} \times 100$$

Where, w_i= initial weight w_f= final weight of samples

2.2.7 In-vivo studies

2.2.7.1 Animals' surgical operation

Albino-Wister rats of 200-250 g of both sexes were purchased from Animal House, IMS, BHU, Varanasi. The rats were acclimatized at least for 2 weeks in the animal facility before surgical operations. The defects (length ~4mm, width ~2mm) were created in the femoral

bones using a high precision dental drill after anesthetizing the animals with Thiopental sodium (20 mg/kg body weight) followed by irrigation of saline. Animals with the defect sites filled with BBG, C2BBG and devoid of materials were evaluated for study of bone healing/ regeneration ability of materials. The animals were provided with ad libitum food and water throughout the study until sacrificed (36^{th} day). After 5 weeks of constant monitoring, the rats were sacrificed on 36^{th} day following the central animal ethical committee's protocol (Protocol No. Dean/2015/CAEC/1421).

2.2.7.2 Radiological examination of defects

Bone defects after 35 days of surgical operations were evaluated using radiographs to study bone remodeling and neo bone formation and healing at wounded sites.

2.5.3 Histology

After 35 days, the rats were sacrificed. The femur bones were collected, washed, and stored in 10% formalin for histology. The bone specimen after decalcification with 10% EDTA stained with Haemotoxylin and Eosin (H&E). The H&E micrographs were examined to assess the formation ability of C2BBG in comparison to both the defect that left untreated and filled with BBG.

Fig 3.1 Some experimental setup. A. FTIR spectroscopy (Bruker Tensor II) B. XRD diffractometer (RIGAKU-Miniflex II) C. Digital pH meter D. Scanning Electron Microscope [ZEISS EVO 18]. E. Universal Testing machine (Tinius Olsen H10KL)