Chapter 3. Materials & Methods

3.1 Materials

3.1.1 Scaffold material-based studies

Type A Gelatin from porcine skin powder with gel strength ~300g Bloom and amorphous chitosan (degree of acetylation: 72.4%) of medium molecular weight (190–300kDa) and 200–800cp viscosity were purchased from Merck, India. Ethanol, acetic acid and chloroform (HPLC grade) were purchased from Changshu Yangyuan Chemical, China. The inorganic salts for SBF were used as received from Fisher. Throughout the experimental study analytical grade reagents and Milli-Q were used as supplied.

3.1.2 Bone cell *in vitro* studies

For the *in vitro* studies, rabbit bone marrow Mesenchymal Stem Cells (MSCs) and rT were purveyed by Institute of Medical Science, Banaras Hindu University (IMS, BHU), India.

For cell culture studies, both low and high glucose Dulbecco's modified eagle's medium (DMEM), Dulbecco's phosphate buffered saline (DPBS), 100x antibiotic-antimycotic solution (AAS), collagenase (type II), 0.25% trypsin/EDTA, Non-essential amino acid (NEA), ß-glycerophosphate (BGP), ascorbic acid-2-phosphate (AA2P), AB, CPC, NaOH were from Himedia, India [184]. Throughout the experimental study when analytical grade reagents and Milli-Q used as supplied. The cells were cultured in growth media [Low

glucose-DMEM supplemented with 1% AAS and 1% NEA] and in the osteogenic induction media [high glucose DMEM, 10% FBS, 10nM dexamethasone (Dex), 10mM ßGP, 1 µg/ml Bone Morphogenetic protein (BMP-2) and AA2P were supplied from Himedia (India)). Lysozyme, HCl, chloramines were purchased from Sigma.

3.2 Methods

3.2.1 Hydroxyapatite synthesis

The Ha used in this study was synthesised by prior published work on wet chemical method [185]. It was prepared based on the following reaction of calcium hydroxide [Ca $(OH)_2$] with orthophosphoric acid (H_3PO_4):

10 Ca $(OH)_2$ +6 $H_3PO_4 \rightarrow Ca_{10}(PO_4)_6(OH)_2$+ 18 H_2O

In this method of synthesis, 100 mL of 0.3 M H_3PO4 solution was added to an equal volume of 0.5 M Ca $(OH)_2$ solution at the flow rate of 3.5ml/min, at room temperature. The pH value of the solution to be precipitated was adjusted to 7.4±0.2 with the addition of 0.01M H_3PO_4.The resultant precipitate was centrifuged in order to separate it from the mother solution. Later, the precipitate was washed with Milli Q and sintered in muffle furnace with temperature adjusted to nearly 700°C for 8h. The sintered white solid stored at room temperature was characterized to identify its crystallinity by XRD and FTIR.

3.2.2 Scaffold Material preparation

3.2.2.1 Constituents Slurry Preparation

Gelatin (Merck) was weighed and added into water and then then mixture was heated upto 40℃ in a magnetic stirrer equipped with heating to dissolve the gelatin and obtain gelatin solutions with increasing concentrations (15 and 25 w/v). The solution (100 ml) was cooled to room temperature by transferring it in the 24-well flat bottom cell culture plate. The prepared hydroxylapatite powder was added at 2.22g/ 10 ml DMF: THF (1:5). Chitosan

(C), gelatin(G) and Ha(H) solution were prepared separately in acetic acid, distilled water at 40°C and in Dimethyl Formamide to Trihydroxyfluoride (DMF:THF::1:5) respectively. Briefly, individual solutions were kept in an ultrasonic bath (Citizen digital ultrasonic cleaner) for 30 min, stirring at room temperature. The obtained suspension was then transferred to the 24-well plates too.

3.2.2.2 Scaffold Material Synthesis

Fibrous scaffolds when prepared from synthesised Ha nanopowder, commercially available coarse gelatin and fine chitosan powder by alternating leaching and freeze-drying [186,187]. Prolonged freezing and drying steps had been incorporated to enable controllable designed optimal freeze-drying of the scaffold material.

In terms of the composition, scaffolds preparation from the raw materials displayed in **Table 3.1**.

Raw material (Merck) Composite pH=7.4	**Initial Concentration (%w/v)**		
	Chitosan/ Chi in 2% acetic acid at RT 5% 'C only'	Gelatine/ Gel in DDW at 40°C 10% 'G only'	Hydroxylapatite/ Ha in DMF/THF (1:3) at RT, 13% 'H only'
Sample	Final concentration in the blend (%v/v)		
CH #1	35	-	40
CG #2	35	40	-
GH #3	-	40%	40%
CGH #4	35%	40%	20%

Table 3.1 Composition of the scaffold materials.

For the reason of controlling the cell viability as close to natural bone, the Ha content in these scaffolds was not exceeded beyond 14%.

In order to prepare 'chitosan loaded in gelatin matrix' (CG), chitosan prepared in situ (1.7 g) was added to the above gelatin solution (25 ml) and slurry was formed as above.

Likewise, in order to prepare 'Ha loaded in gelatin matrix' (GH), Ha slurry (5ml of 2.2g/10ml) added to the above gelatin solution (25 ml) and slurry was formed as above. Every scaffold was then stored in the culture well wherein they were fabricated to be used immediately for characterization and other studies.

Three vials of 20 ml each were filled with 0.5 g polar gelatin per 2 ml Milli-Q water formed slurry when placed in the ultrasonic bath (Citizen digital ultrasonic cleaner) after 2h stirring at 40°C. Total 3x20 ml of 5% (w/v) chitosan / 2% acetic acid solution was prepared as slurry. This slurry was added to every scaffold which will contain chitosan as one of their component [186]. Following degassing after continuous 1h agitation, the solution made from chitosan and gelatin was ready for the 24 well cell culture treated plate (Himedia) and was labelled as CG. When Ha dissolved in a solution of 'Dimethyl Foramide (DMF): Trihydroxy Fluoride (THF) :: 1:5' was co-precipated with 'CG' solution to be fabricated into the scaffold it was labelled as 'CGH'. Likewise, when Ha in DMF: THF was co-precipitated with aqueous gelatin solution to be fabricated into the scaffold it was labelled as 'GH'. Each solution when crosslinked by the addition of 0.25% glutaraldehyde at 37 °C for 1h before the pH was adjusted to 7.4 and freezing done at -20 °C for a minimum of 12 h before being lyophilized at -40 °C at 40mm torr for 72 h. For storage and characterization, the lyophilized scaffold (10mg, 22mm diameter and 10mm height) was sterilized by immersing in 70% ethanol followed by UV exposure for a minimum of 20min each.

Gelatin-matrix was loaded with either chitosan or hydroxylapatite to prepare scaffold material variants to be optimized for cell studies. The four scaffold material variants studied in this research involves CG, CH, GH and CGH.

This research study was performed for over a period of 28 culture days among four composite groups on the basis of raw material constituting part of the composite: CG (chitosan in gelatin matrix), GH (Ha in gelatin matrix), CH (Ha in chitosan) and CGH (chitosan and Ha in gelatin matrix). Composition of the composites have been summarised in **Table 3.1** Composite based composite slurry was prepared, and then lyophilised at 40mm torr at -40°C. The groups were studied *in vitro* for morphological, mechanical and physicochemical characterization.

3.3 Scaffold Material studies

Here, the key characteristic of the successfully synthesised BTE scaffolds were critically analysed.

3.3.1 Morphology analysis

3.3.1.1 Surface morphology

Surface contact stimulates the 1st response for the interacting cell. Atomic force microscopy (AFM) analysis derived 2D and 3D surface topographies for the scaffold materials were illustrated. The distinctly denser surfaces in terms of average roughness have been defined for the scaffold materials.

For AFM both the harvested and seeded scaffold materials were mildly washed in DPBS (pH 7.4) and fixed on the 22mm coverslip using 0.5% gelatin. The surface roughness parameters of every scaffold including roughness average, root mean square roughness, surface skewness, and surface kurtosis were measure from nanoScope III A multimode AFM (NT-MDC, USA) processed by Nova Analysis software [87].

3.3.1.2 Core morphology

The core morphology was characterized using Scanning Electron Microscopy (SEM) (Quanta 200 F, FESEM, USA) at an accelerating voltage of 5 kV. SEM image of each scaffold and cell-scaffold sample was analysed for elemental mapping with Energy Dispersive X-ray (EDX) spectrometer equipped with SEM.

For SEM-EDX, harvested and seeded composite was mildly washed in PBS (pH 7.4), fixed overnight by 0.2M Sodium cacodylate in osteogenic media at 4°C, later at room temperature dehydrated with graded increasing ethanol concentration (10%, 30%, 50%, 70% and 100%).

Samples were fixed in sodium cacodylate before gold sputtered at 10mA for 5 min (Quarum Q150RES Penta FET Preunion) for SEM (ZEISS EVO/18 Research) for SEM. For better conductivity the samples placed on carbon tape were coated with gold particles under vacuum before being examined by SEM-EDX. SEM equipped EDX did spectrum analysis for calcium to phosphate molar ratio of the samples. Moreover, SEM was done to observe the microstructure's morphology and cell growth in interior walls of the scaffolds. TEM-SAED (HRTEM 200KV HT (electron beam) FEI TECHNAI G^2) was done to find uniformity in pore morphology and the crystal spots presence in the different type of biomaterials designed. For TEM-SAED, sample was prepared by heat-drying the sample placed on the 300 mesh carbon chips in vacuum-oven for 15-20min.

3.3.2 Crystal phase Analysis

3.3.2.1 Qualitative Analysis of Ca-P deposits

The crystal and chemical phase distribution in the lyophilised composite, both before and ECM distribution after bone cell-seeding was determined from XRD (Rigaku D/Max-III X-RAY DIFFRACTOMETER with DIFFTECH software) using 0.717 nm Mo radiation

generated at 40 kV and 30 mA. The sample was scanned at 2°/min from angular range 10°to 60° in 2θ degrees.

The samples for XRD analysis were made into fine-powder form in a mortar-pestle.

3.3.2.2 Quantitative Analysis of Ca-P deposits

Composition dependent intermolecular interaction of the bone cell deposited and mineralized extracellular matrix (ECM) was studied by FTIR spectrophotometer (IRAffinity-1S SHIMADZU FTIR) at a spectral resolution of $4cm^{-1}$ from 4000 cm^{-1} to $500cm^{-1}$. FTIR spectrum of the samples were obtained from translucent fine pellet discs containing 1-part sample in 75-part Potassium Bromide (KBr). Background data was subtracted for samples at n=5 was analysed and spectra for the runs were averaged.

3.3.3 Thermal behaviour

The thermal properties (DSC 6000-Perkin Elmer) of the scaffold determine by heating each of them at 5°C/min in the temperature scan range from 0 °C to 400 °C. This scanning then done in nitrogen environment at a flow rate of 20.0ml/min.

Calibrated DSC using indium as standard and an empty pan as reference were run under preset thermal scanning conditions. A sample of 10 mg was hermetically sealed in a aluminium DSC pan of 100 µL. The thermal scanning conditions: heating from 25 to 400°C at 5°C/min, holding at 400 °C for 5 min, cooling from 400 to 20 °C at 20 °C/min.

Differential Scanning Calorimetry (DSC) was done in Pyris 6 DSC's DSC 6000 to study the thermal behaviour of the biomaterial composites after fixing the seeded scaffold with sodium cacodylate same as in SEM-EDX.

3.3.4 Uniaxial Compressive Strength (UCS)

The UCS of cylindrical composite (22mm diameter x 11mm length), were determined using TA5 cylinder (12.7mm Diameter and 35mm Length) by Texture analyser using Brookefield's CT3 10K, with a 9.81N load cell.

Five different samples were tested to determine the average stiffness Young's modulus.

The slope of the initial linear-portion was calculated as the elastic modulus from the stress–strain curve. The specimens from each scaffold were tested (n=5), and the data was represented as the average of the three measurements.

3.3.5 Porosity

The porosity of 22x11 mm CG, GH, CH and CGH composite based composites was studied by liquid displacement after immersing them in 5ml 99.9% ethanol for 7h in the graduated cylinder.

The composite porosity (ε) was measured as:

$\varepsilon\ (\%) = (V_1 - V_3)/(V_2 - V_3)\ X\ 100.$

Where, V_1 is the initial ethanol volume, V_2 is the volume of ethanol soaked composite after 7h and V_3 is the residual ethanol volume after composite removal from the cylinder [188].

3.3.6 *in vitro* degradation

The biodegradation of the lyophilised 22x11 mm CG, GH, CH and CGH composite based composites was studied by immersing them in lysozyme (10,000 U/mL) in DPBS (pH=7.4) at 37°C with 5% CO_2. After 7 days, the composites were washed in MilliQ for removing the surface ions and lyophilised for SEM and TEM-SAED.

The average degradation rate of the composite was computed as follows:

Degradation % = $(W_i - W_d) / W_i\ X\ 100$ [122]

Where, Wi is the initial dry weight before immersion and Wd is the sample dry weight measured after drying post-degradation. The degradation rate was noted as the mean±SD

(n=5). The degradation-study was carried out till culture day 28. Among the four groups, the scaffolds sufficiently stable had been used further for *in vitro* bone tissue regenerative studies.

3.3.7 *in vitro* biomineralization

The biomineralization of 22x11 mm CG, GH, CH and CGH composite based composites was studied by immersing them in 1.5x SBF solution, pH 7.4 at 37°C with 5% CO_2. The SBF solution was prepared as reported in previous work [157].

Sr. No.	Components	Concentration (g/L)
1.	NaCl	0.136M
2.	HCl	40ml*
3.	$NaHCO_3$	0.001M
4.	KCl	0.009M
5.	$K_2HPO_4.3H2O$	0.0027M
6.	$MgCl_2.6H2O$	0.0049M
7.	$CaCl_2.2H2O$	0.0018M
8.	Na_2SO_4	0.0010M

Table 3.2 Composition of the prepared Simulated Body Fluid (SBF)

*Specific gravity of HCl= 1.18 g/ml

Scaffolds from every group were incubated at n=5, for 28 days in 2.5 ml of 1.5x SBF in the 24 well cell-culture plates. After every 7 days, composite samples were washed with 3X Milli Q for removing surface minerals, and later again lyophilised for morphology and ability of apatite formation examined by SEM and TEM-SAED, respectively. Among the four groups, the most bioactive scaffolds had been used further for *in vitro* bone tissue regenerative studies.

3.3.8 Antibacterial activity

The innate antimicrobial properties of the scaffold materials namely CG, CH, GH and CGH were evaluated *in vitro.* After scaffold implantation this property limits the development of infection at the 'critical-size defect' site. The microdilution method using 9cm-diameter cell culture tested petri-plates, according to Microbial Type Culture Collection (MTCC), Chandigarh against the following reference bacterial strains: *Staphylococcus aureus* (MTCC 7447), *Bacillus subtilis* (MTCC 6633) and *Psuedomonas aeroginosa* (MTCC S1) was followed. Fresh liquid culture for every test microorganism was used for the test performed with n=5. The dilution of the bacterial suspensions was performed to adjust the microbial concentration to 1.0×10^7–1.0×10^9 CFU/mL. The minimum inhibitory concentration (MIC) of the scaffold material was the lowest concentration that caused 100% inhibition of microbial growth while Minimum bactericidal concentration (MBC) was the lowest concentration resulting in >99.9% reduction of the initial inoculum.

One loop-full of the bacteria suspension was inoculated in the petri-plates using the L-shaped spreader and incubated in an air-bath shaker at 37 °C for 24 h. The cultures of three strains containing approximate 10^7 CFU/mL were prepared and used for the evaluation of antibacterial activity. Scaffold samples were cut-out as 5mm discs and sterilized before being placed in the hole made in the bacterium-inoculated agar. Again plates were incubated at 37 °C for the antibacterial activity and the inhibition zones were measured with the transparent scale and estimated once daily till 7 days.

3.4 Bone osteoblast studies

3.4.1 Animal care and bone defect introduction

All animal treatments and procedures were approved by the Central Institute Animal Care and Research Advisory Committee of Banaras Hindu University (BHU) in accordance with international guidelines. Adult male rabbits weighing 2-3Kg were housed in individual stainless-steel cages (pathogen free conditions) under standard temperature conditions (22°C) and hygrometry (40–60%) with a 10-h to 14-h light-dark cycle (lights on between 8 am- 6 pm). Rabbits had free access to water and standard laboratory feed (pellet).

Before cell-seeding all the biomaterials samples (22x11mm) were sterilized for in vitro studies in the 24-well plates by the same method as done for their characterization studies. Also, the cell culture and in vitro assays samples incubated in physiological conditions only.

3.4.2 Cell isolation

Rabbit bone and bone marrow samples were supplied by the Institute of Medical Science, Banaras Hindu University (IMS, BHU) India. Both rT (enzymatically with brief modification) [189] and rM (ficoll gradient separation) isolated [166], counted and expanded in the culture .

The osteoblast from the bone tissue were isolated by carrying out the enzymatic digestion of the bone tissue, involving incubation of the harvested bone, possibly contaminated with the muscle in the sterile enzyme solution (1.5% U/ml collagenase type I in 0.25% trypsin-EDTA) for digestion in physiological condition for 15 minutes. The first two of the five digests were discarded. Every enzyme initiated digestion step was intermittent with HBSS wash. As the last digest was highly populated with osteoblast, it was used for cell counting and for further cell studies.

Additionally, the MSC from the bone marrow of the anterior iliac crest were isolated after layering 500µl of marrow over Ficoll-Plaque PREMIUM centrifugation medium (From Himedia, India). It was centrifuged at 400g for 15 minutes. The layer containing the mononuclear cells was transferred to a sterile microcentrifuge tube containing sterile DPBS. 0.01%-0.001% of MSCs was enriched by both positive and negative selection using antibodies to cell surface markers correlated with the MSCs to be expanded *in vitro* [190]. Positive and Negative selection employs monoclonal anti-CD90-FITC, anti-CD105-FITC and anti-CD73-FITC antibodies against CD90, CD105 and CD73, respectively and the hematopoietic cell population depletion by using monoclonal anti-CD34-FITC and anti-CD45-FITC antibody (Sigma) against CD34 and CD45 surface markers, respectively [129]. Both the cell samples were stored in a Freezing mixture until counted and cultured for seeding on the scaffold materials for further studies.

3.4.3 MSCs Identification

The monoclonal antibodies (mAbs) were directly conjugated to fluorescein isothiocyanate (mAb-FITC) for immunofluorescence staining. The stained cells were analyzed on the FACScan flow cytometer (BD™FACSCalibur) along with Cell Quest software to analyse the data. The level of marker expression was the analyzed from the mean fluorescence intensity (MFI) of the cells sampled. To determine changes in surface markers during differentiated cells monolayer culture, fold change in the level of expression of the markers at different culture time were calculated in relation to the beginning of the culture. A two-tailed, unpaired Student's t-test was done to define significant change in expression (fold change >2) with a P value < 0.05.

The combinations of changes in gene expression of the ECM components which will represent the differentiation status of MSCs were calculated from the ratios of mRNA levels of ECM components (collagen I, osteocalcin and BMP-2) [180].

3.4.4 Cell culture

MSCs were stored at equal concentration in the Freezing mixture and later thawed to culture them in 'T25 cell culture' Flask with complete Dulbecco's Modified Eagle's Medium low glucose (DMEM-LG) supplemented with 10% Fetal Bovine Serum (FBS), 1% antibiotic-antimycotic solution (AAS) and Non Essential Amino-acid (NEA) (Merck, India). 'rOb obtained from differentiated MSCs' (rM) were culture in Osteogenic induction media to promote differentiation (high glucose DMEM, 10% FBS, 10nM Dex, 10mM ßGP, 1 µg/ml BMP-2) kept in 5% CO_2 at 37°C and water-saturated atmosphere [171]. DMEM was already supplemented with sodium phosphate, Glutamate and Ascorbic acid essential for promoting the osteogenic phenotype in the progenitor cell / MSCs. To the cell plates reaching near confluency, equal volume of DMEM and 0.25% Trypsin-EDTA was used to harvest freshly isolated cells [191] through the successive passages, which were to be used for the *in vitro* assays.

3.4.5 Cell cycle analysis

Both rT and rM (1×10^4 cells/250µl/well) were grown in the 22mm diameter 24-well cell culture plates. Cell culture was trypsinized, fixed in ethanol and labelled for staining with Fluorescein Isothiocyanate (FITC) and propidium iodide (PI) in sets of five (n=5). Flow cytometry of the cells was done by FACScan flow cytometer (BD™FACSCalibur) along with Cell Quest software to analyse the data. The cell count obtained from the Flow cytometry gave the distribution of the number of cells present in each cell cycle phase.

3.4.6 Growth curve analysis

The population doubling time (PDT) and specific growth rate of both the cell types, rM and rT were determined from the passage 4(P4) cells at 1×10^4 cells/250µl/well cultured in the 24 well plates.

Growth curves were followed from the daily cell count of the number of adherent cells 5 weeks, and the PDT was determined from the number of adherent cells counted at the start and end of every passage.

The culture plates were incubated under physiological conditions with change in growth media thrice a week for 5 weeks. From the average cell count the semi-log curve was plotted against culture duration to generate the growth curve.

The PDT was calculated as per the following equation described by A.Somal et. al.[192]

$$PDT = \frac{t \log 2}{\log \frac{Nt}{N_0}}$$

Where, t depicts the total culture duration in hrs, N_0 and Nt depicts the initial cell number and cell number at particular culture duration.

3.4.7 Scaffold sterilization and conditioning

Scaffolds (22 mm in diameter, 11mm in height) kept in the 24-well plates wherein they were freeze-dried synthesised, were sterilised after immersing them for 15-10 min in 70% (v/v) ethanol. For conditioning, scaffolds were incubated in growth media for 2h at '37°C in a humidified atmosphere of 5% CO_2' (**physiological conditions**). The medium was discarded and replaced with fresh before the scaffold were tested via cell-seeding experiments. Scaffolds were tested in sets of five (n=5) in all the experiments. Both the conditioning and sterilization steps did not introduced any biological and chemical modification.

3.4.8 Osteoblast cell-seeding

The sterilised and conditioned scaffolds kept in the 24-well plates were seeded with either rM or rT. The cell population from the passage 4 of both the cell types was used as the donor population for the in vitro experiments. The scaffolds GH and CGH were made ready to use for *in vitro* studies by sterilisation and conditioning in Biosafety level-II in a laminar

flow cabinet. For cell seeding, the used media was replaced with the cells at $4x10^5$ /ml cell suspended in the 250µl fresh warm media and kept under physiological conditions for up to 28 days. The medium was changed thrice a week and cells-in-scaffolds were isolated for *in vitro* assays at culture day 7, 14, 21 and 28. Scaffolds were carefully rinsed with DPBS intermittently with media change. Scaffolds were tested at n=5.

3.4.9 Sample pre-treatment

Each cell-scaffold sample was rinsed with PBS (3x) and incubated in a solution of 0.25% trypsin and 0.005% collagenase, under physiological conditions. The samples were centrifuged and the pellet of cells were suspended in fresh media. These steps were repeated thrice, to remove all of the seeded cells the scaffold. Then, the total cell concentration was determined to assess cell adhesion efficiency in the scaffolds by counting with a Coulter Counter Z1 (Beckman).

The culture media was replenished thrice a week to avoid suppression of cellular activities due to nutrient exhaustion and metabolic waste accumulation. Cells were trypsined prior to seeding at the initial cell density of 10^5 cell/ml on scaffolds.

To eliminate residual ethanol and improve the cell attachment, The scaffolds were rinsed with cell culture tested DPBS (2x), transferred to a 24 well cell culture-treated polystyrene plate and incubated under physiological conditions for 2-4h in the growth cell culture medium.

3.4.10 Osteoblast and its matrix

The morphology of both the cell types, both before and after seeding was visualised in its native environment by Confocal Laser Scanning Microscopy (CLSM). Both the rT nd rM seeded scaffolds were fixed by a droplet of 4% (v/v) formaldehyde, at 37°C for 5 min. The samples once fixed were incubated with Hoechst 33258 and 1µg/ml Alexa-Fluor 488 to stain the nuclei and ECM, respectively defining the degree of cell proliferation and thereby migration at room temperature for 15 min in dark condition. In DAPI staining, dye-positive living cells were evaluated for synchronous population to be studied in the scaffold by FACScan flow cytometer (BD™FACSCalibur) along with Cell Quest software. From every experimental group (n=5), washed intermittently in PBS (pH=7.4) replacing DMEM and fixed. All sample groups permeabilized with 500µl of 0.025% (v/v) Triton in PBS for 5 min. The 2 mL of 1% (v/v) FBS also in PBS used as the stopping solution for 20 min. For 5 min in the dark, 5 µL Hoechst 33258 in PBS at 1:100 ratio was added to each sample. Lastly, samples were dried after 3X PBS wash to view in CLSM [193]. Each sample was imaged (Carl Zeiss LSM 780) in five random regions under focus at 63X [194].This defines cell morphology after seeding the composite via fluorescence indicating the loci of new bone deposition. FACS's flow cytometry quantifies the cellularity of the composite in the experimental groups [165].

3.5 Scaffold Material-Bone cell studies

3.5.1 Sample treatment

Scaffolds were sterilized in the flat bottom 24-well culture plates wherein they were freeze-dried synthesized [195]. All samples of equal weight (10mg) were sterilized by briefly immersing them in 70% (v/v) ethanol absolute (Merck).

Before cell seeding, the scaffolds (n=5) were sterilized and supplemented with 100 µl osteogenic media at 5% CO_2 in an CO_2 -Incubator for 2 hours at 37°C, in the culture plates. This research study was done over a period of 5 weeks among scaffold groups: GH and CGH (without rOb) and four experimental groups (on the basis of the origin of rOb-seeded in it): rMGH, rMCGH, rTGH and rTCGH as summarised in **Figure 3.1**. GH and CGH were chosen for cell seeding on the basis of optimization of the scaffolds group.

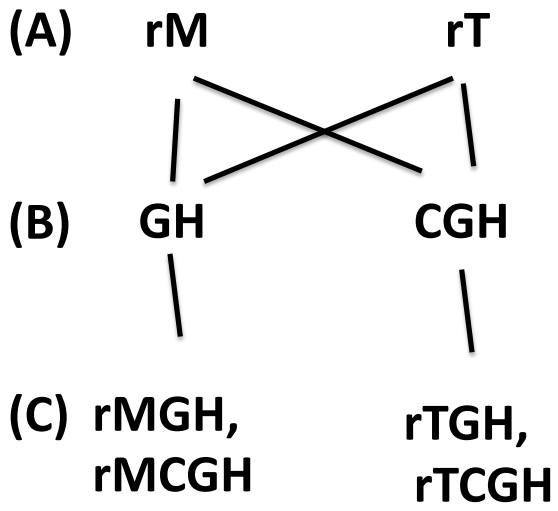

Figure 3.1 Schematic representation of the osteoblast type-scaffold group studied: (A) Osteoblast cell selected: rM and rT, (B) Scaffold type optimised and selected: Gelatin and hydroxyapaptite based scaffold [GH] & Chitosan, gelatin and hydrosyapatite based scaffold [CGH] and (C) Osteoblast seeded in the scaffold type: rMGH and rMCGH stands

for rM seeded on GH and CGH, respectively. Likewise, rTGH and rTCGH stands for rT seeded on GH and CGH, respectively.

Each group after seeding with rabbit cells was studied for their morphology and *in vitro* properties. With a sample size of n=5, regular sampling was performed every week for up to 5 weeks for *in vitro* assay

These moistened scaffolds were then seeded with thawed osteoblast at a concentration of 10,000 cells/250µl media/well. Cell culture was carried out till ~70% confluency in monolayer was observed up to 4th Passage and retrieved at culture day 7, 14, 21 and, 28 for biomaterial based *in vitro* studies, also done in the culture plates.

For static seeding, small multiple scaffold pieces were seeded with a high concentration of cell suspension, to ensure that a large cell population had full access to the scaffold structure. The scaffold has the ability to allow cell migration, infiltration and attachment, both from out-to-inside the scaffold and within the scaffold cell itself.

3.5.2 Surface Roughness

Surface attachment properties of each biomaterial immediately after rOb cell seeding was reported from AFM. AFM images showing contact surface topography differences were recorded using nanoScope III A multimode AFM (NT-MDC, USA).

3.5.3 Micro-morphology

SEM was done to examine the *in vitro* morphology (pore structure) of the scaffold materials and cell attachment (Day 21) on the framework. The samples were gold sputtered at 10mA for 5 min (Quarum Q150RES Penta FET Preunion) for SEM (ZEISS EVO/18 Research). The elemental mapping of each sample spectrum was analyzed with energy dispersive X-ray (EDX) spectrometer by SEM equipped with EDX.

On culture day 21, one scaffold each from rMGH, rMCGH, rTGH and rTCGH was used for SEM analysis. The cell-scaffold construct was taken out of the culture plate and rinsed in DPBS (2x) and prepared for SEM same as in section 4.3.2.2 before sputter coated with gold for SEM observation.

3.5.4 Cytocompatibility

To validate that the scaffolds *in vitro* in terms of adequate cellular development, the proliferation of rM and rT within the scaffolds when cultured in both the growth medium and in the osteogenic differentiation medium was studied.

There was no need to replenish the culture medium immediately before the *in vitro* assays. Dark 24 well Flat-bottomed cell culture plates were used for checking fluorescence-based cytocompatibility in these scaffolds by bottom-reading. BioTeK SYNERGY/H1 equipped with a multimode microplate reader was used to assess measurements.

The response of viable cells in a 22x11 mm scaffold was compared w.r.t the control sample i.e. cell cultured on the 24-well flat bottom plate surface. Experiments were performed in sets of five (n=5).

3.5.4.1 DNA

Hoechst 33258 was also used here to measure total DNA content. Supernatant from the cell-lysate was centrifuged at 400g for 15min before incubating it in 1 µg/ml Hoechst 33258 also under physiological conditions. Fluorescence of the samples was measured at both 360 and 460nm.

3.5.4.2 Alamar Blue (AB) fluorometric Assay

Fluorometric Alamar Blue (AB) Assay was done to analyze the rOb cytocompatibility when cultured with the scaffold materials. ABA quantitatively determines the viable cell

count cultured in the scaffolds. In the presence of AB, the live cell initiates chemical reduction of resazurin (blue) to resorufin (red). Fluorescence (excitation, emission at 530nm and 590nm, respectively) based cell intensity indicating the chemical reduction reaction was read every hour until 8h. The cell cytocompatibility was assess using non-corrosive AB fluorometric assay at culture day 7, 14, 21 and 28 after seeding. Supernatant with AB and without cells, autoclaved supernatant with AB mix and supernatant with cells but without AB used as negative, positive and untreated control respectively.

Evaluation of cell viability and proliferation			
Test	Histological evaluation	ABA (Cell Viability)	DNA (Cell proliferation)
Procedure	Sample Preparation	• Same 'Cell on scaffold' samples were used for every ABA till culture day 28.	• 'Cell on scaffold' samples were lysed in 0.02% Triton X 100. • Lysate was centrifuged at 4000 rpm, 15min
	Assay	• Incubation in 10% Alamar Blue (in DMEM) • Fluorescence was measured at 530nm (excitation), 590nm (emission).	• Supernatant incubated in 1 µg/ml Hoechst 33258, 37°C. • Fluorescence was measured at 360nm (excitation) and 460nm (emission).

Table 3.3 Sample preparation and assay steps followed for Hoechst33258 and Alamar Blue based Assays.

The supernatant from each cell-biomaterial sample incubated with 10%AB for 3-5h until change in colour was visible. Fluorescent AB based Assay was perform to determine percent cell viability. Initially, cell-seeding at $5X10^4$ cells/well concentration were culture with change in media twice in a week. The chemical reduction reaction after 3-5 h read using BioTeK SYNERGY/H1 equipped with a multimode microplate reader at the culture

day 7, 14, 21 and 28. The Alamar readings quantified the scaffold system with fluorescence excitation at 530nm and emission at 590nm.

3.5.5 Mineralization

3.5.5.1 Alkaline Phosphatase (ALP) based assay

Quantitatively ALP determines the early-differentiation marker of the bone cell [196]. The osteogenic cells seeded on every biomaterial placed in the 24 well cell culture plates at $5X10^4$ cells/well seeding density. Sampling was done on culture day 7, 14, 21 and 28.

Evaluation of mineral deposition			
Tes	Histological evaluation	ALP	Alizarin Red S(ARS)
Procedure	Sample Preparation	• Cell in scaffold samples were lysed with 0.025% Triton X in DPBS in the 24 well plates.	• Cell in scaffold were rinsed with DPBS (2X). • Fixation in ice cold 70% etOH, 1h, 37°C • 3X Milli Q wash
	Assay	• 10 µl Cell Lysate incubated with 10mM ALP-substrate (p-nitrophenylphosphate in 50% ddH2O:50% buffer, 2-amino-2-methyl-1-propanol (AMP), pH=10.2) in dark. • Incubated at 37°C, 5% CO_2 until sufficient color was seen. The	• 20mM ARS, 1 h, 37°C, Dark (Incubation) • 5X Milli Q wash • 10% Cetyl-pyridinium chloride (CPC), 1 h • Absorbance at 540nm

		reaction was stopped with 250 µl of 0.3 M Na_3PO_4. • Absorbance was measured at 405nm.	

Table 3.4 Sample preparation and assay steps followed for Alkaline Phosphatase and Alizarin Red S.

3.5.5.2 Alizarin Red S (ARS) based assay

The differentiation of MSCs into osteoblastic lineage was assessed from ARS [171]. The calcium deposition or late-mineralization condition was indicative of the complete differentiation of rOb. This deposition was measured by ARS, to quantify rOb mineralization after growing cells in different scaffold biomaterial after induction of osteogenesis.

3.5.6 RNA isolation and real-time PCR

Collagen type I (colI), osteocalcin (OC) and *bone morphogenic protein-2 (BMP-2)* gene expression in rOb seeded scaffolds was evaluated quantitatively in Real-Time Polymerase Chain reaction (Q-PCR). The cell seeded scaffolds were cultured *in vitro* and harvested for Q-PCR at culture day 7, 14, 21 and 28. The cell-scaffold hybrid was washed in PBS before freezing in liquid nitrogen followed by immersing in TRIzol™ reagent (Thermofischerscientific) to isolate RNA. High capacity cDNA Reverse Transcription Kit was used to convert the isolated RNA to cDNA. Here the SYBR GREEN JUMPStart (Sigma) was used in the gene expression analysis from cDNA for *Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), colI, OC and BMP-2.* The reaction was started using

5µl cDNA, 0.8 µl primer and 20µl PCR mastermix. The reaction was run for 30 cycles using Applied Biosystems (ABI) Step One instrument and the data was analyzed using ABI software. The 'relative gene expression' was normalized by the mean cycle threshold (Ct) value of *GAPDH* as the house keeping gene for each expression performed in sets of five (n=5). The $2^{\Delta\Delta ct}$ relative quantification defined the method for this analysis [197]. The primer sequence of these genes is listed in **Table 3.5**.

Sr.No.	Gene	5'→3'	Sequence	Accession Number
1.	*GAPDH*	Forward	CATCATCCCTGCCTCCACTG	NM_001082253
		Reverse	GATGCCTGCTTCACCACCTT	
2.	*BMP2*	Forward	5'GGACGACATCCTGAGCGAGT	NM_001082650
		Reverse	5'CGGCGGTACAAGTCCAGCAT	
3.	*Osteocalcin*	Forward	5'TTGGTGCACACCTAGCAGAC	XM_002715383
		Reverse	5'ACCTTATTGCCCTCCTGCTT	
4.	*Collagen type I*	Forward	5'GCCTGAGGGTTTTATAAAGG	NC_013678.1
		Reverse	5'TTAGCTCTGTGGGGTGTAC	

Table 3.5 Primer Sequence of gene markers promoting osteoblastic lineage in rabbit osteoblast cells.

3.5.7 Immunoblot

ColI, OC and BMP-2 protein expression in culture Day 14 rOb seeded scaffolds were evaluated from immunoblot. The cell seeded scaffolds cultured *in vitro* and harvested for immunoblot at culture day 14. The cell-scaffold hybrid washed in PBS followed by immersing in TRIzol^{TM} reagent (Thermofischerscientific) to separately isolate colI, OC and BMP-2. Anti-collagen antibody, anti-osteocalcin antibody, anti-BMP-2 antibody and anti-GAPDH antibody were purchased from Merck.

3.5.8 Biochemical Analysis

3.5.8.1 Hydroxyproline based Collagen assay

The collagen content was estimated by the modified hydroxyproline assay (using the hydroxyproline to collagen ratio of 1:7.69) based on hydrochloric acid from previous work [198]. From each experimental group five cell-scaffold samples were analysed at 570nm using a Synergy™ HT (BioTek Instruments Inc.) multi-detection microplate reader.

3.5.8.2 Osteocalcin assay

Osteocalcin (OCN) an osteoblastic-markers was selected to confirm the osteogenic characteristic of the cells established by the scaffold when seeded with both the rabbit cell lines separately *in vitro*.

The osteocalcin was quantified by enzyme immunoassay according to manufacturer's instructions. Briefly, osteocalcin antibody, horseradish peroxidase-conjugated antibody was added successively to 150 µl cell culture medium. Tetramethylbenzidine was used as the substrate. The reaction was stopped by adding 0.5M H_2SO_4.

From each experimental group five cell-scaffold samples were analysed at 450nm using a Synergy™ HT (BioTek Instruments Inc.) multi-detection microplate reader. Osteocalcin concentration was expressed as pg/ml.

3.5.8.3 Glucose diffusion analysis

3.5.8.3a Measurement of the D value

Two slots in the 12-well cell culture plate were used for single-point data collection. Each well was 7.3cm^3 in volume (dimension of the cylindrical well: 2.2cm diameter X 1.9 cm depth). Each well can hold up to 1.2ml media volume.

The wells were incubated with cell culture media saturated 'cell-scaffold' combination. The set-up was kept at 37ºC. Glucose at 1mg/ml was added to saturation in one in every two wells. The 12 well plates were incubated in the CO_2 incubator.

As per Fick's law, Diffusion flux, J is directly proportional to the concentration gradient of the particle ($d\Phi/dx$). Provided there was no change in the volume of the diffusion cell.

$$J = -D \frac{d\Phi}{dx}$$

$$= -DA \Phi_d - \Phi_r / x$$

Where, J is the diffusion flux, mass transfer through an area per unit time,

Φ is the concentration of the diffusing solute,

x is the scaffold thickness,

A is the area of the scaffold,

D is the effective diffusivity of glucose in the seeded scaffold and

V is the volume of the well.

D was calculated by fitting the experimental values into the equation for J. All experiment was performed at n=3. Also, no significant deviation among the data values were recorded.

3.5.8.3b Measurement of D value for cell-scaffold saturated cell culture media

A UV spectrophotometer () was used to record the change in glucose concentration w.r.t time at 190nm. Each well was filled to saturation using 1.2 ml of glucose (1mg/ml). Sampling was done from both the wells, with and without cell-scaffold saturated with water after 1,2,4,6 and 8 hours. All the readings were recorded in triplicate.

3.6 Statistical Analysis

Both quantitative and qualitative studies then performed reproducibly (with n = 5), and findings expressed as mean±SD. P value ($P < 0.05$) considered to be statistically significant in one-way ANOVA using GraphPad Prism 5.

3.7 *In vivo* osteoblast studies

The bone cells used in the study were isolated from the 'critical-defect site' tissue obtained from the healthy donors (n=5). All surgical procedures were approved by the Central Institute Ethical Committee at Banaras Hindu University, India. Twelve healthy mature 4-month-old male white rabbits around 2-3Kg, were studied in groups (n=3). All the surgical steps were performed under aseptic physiological conditions. The rabbits were anaesthetized at Day 0 by administering Ketamine hydroxloride (22 mg/kg of body weight) and Xylazine (10 mg/kg) intramuscularly. The left-hind limb was shaved to expose the area on femur, prepped with 1.5% betadine and 70% alcohol. The defect was created by drilling a 2mm diameter hole and subsequently increasing the defect size to the size of scaffold to be implanted (22mm diameter) under saline irrigation to cool and remove the residual tissues. To achieve homeostasis at the defect site, sterile gauss was used to blot blood. All the implantations were carried under aseptic conditions. Schematically the steps in the *in vivo* have been summarised in **Figure 3.2**.

Sterilized scaffolds (22mm in diameter, 11mm in height) were conditioned 1 day before the bone defect surgery. Scaffolds were dried carefully by absorption with a sterile compress before the implantation.

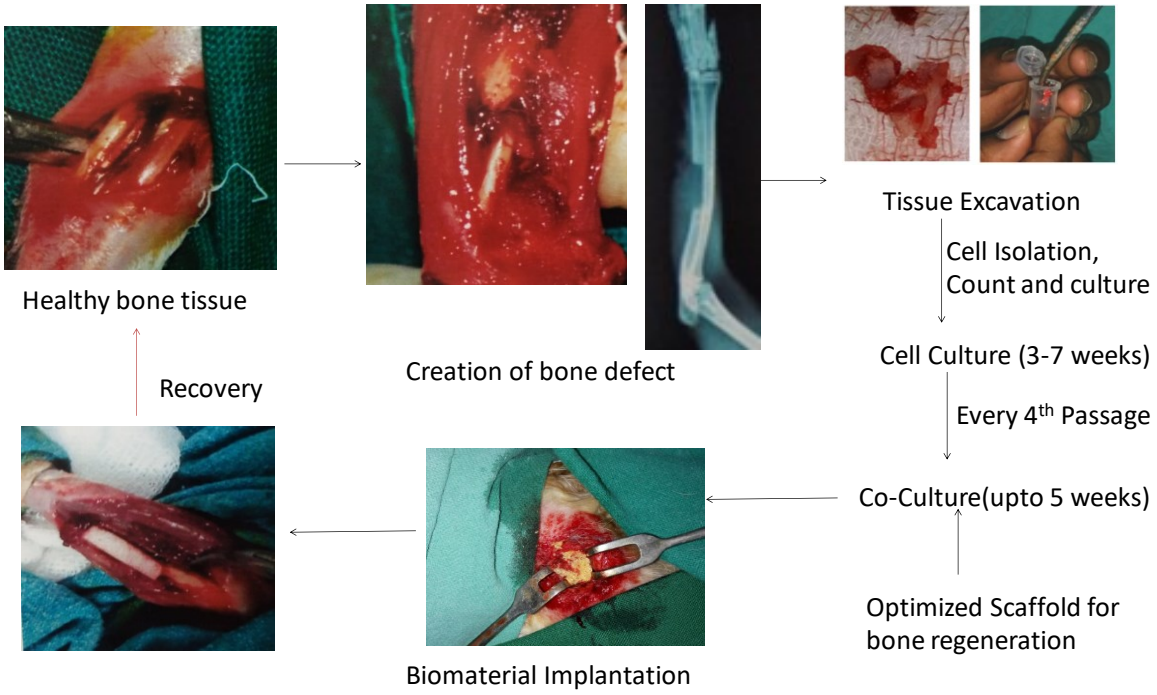

Figure 3.2 Schematic display of the *in vivo* study carried out for rabbit tissue derived osteoblast.

Each (n=5) 1/3rd of the rabbit population was implanted with rMCGH and rTCGH scaffolds into the defect site, while in the last 1/3rd of the rabbit population the defect was left empty and was considered as the control group. The rabbit skin and muscle were sutured (VicrylVR 4/0) followed by recovery from anesthesia after the intramuscular injection of atipamezole (0.5mg/kg). Bilateral surgery performed in the femur as the approach was same for all the rabbits. As the postoperative care, all animals were administered with analgesia for consecutive three days. At predetermined time points (2, 4, 8 and 12 weeks), animals from different groups were euthanized by carbon dioxide inhalation and the femoral end of ulna was dissected for further analysis.