Chapter 3: MATERIALS AND METHODS

3.1 Materials

PCL (Polysciences.Inc., Warrington, PA) with a number average molecular weight of 43,000-50,000 was used for this study. The nanofillers nSiO$_2$ (10nm) and halloysite nanoclay were purchased from Sigma, USA and nHAp as well as MF-nHAp were 30-40nm particles synthesized in our laboratory, details can be found in paper by Anusha et al. Chloroform (Merck, India) and methanol (Merck, India) were used as solvents for electrospinning. Dichloromethane (Merck, India) was used for intercalation of NC in PCL. For hMSC culture, Iscove's Modified Dulbecco's Medium (IMDM), MSC specific fetal bovine serum (MSC-FBS), penicillin-streptomycin and Trypsin-EDTA were all purchased from Invitrogen, USA. Cell viability and proliferation assays were done with alamarBlue (Invitrogen, USA). Proliferation assays also involved MTS (Promega, USA) and PicoGreen™ (Invitrogen, USA). For fixing cells before analyzing samples under microscope, gluteraldehyde (Fluka, USA), methanol or paraformaldehyde (Sigma, USA) were used. Serum protein adsorption assay was done using bicineconic acid (BCA), copper sulphate and bovine serum albumin (BSA)(Sigma, USA) and for testing the ALP activity, ALP enzyme (HiMedia, India) and p-nitro phenol phosphate liquid substrate (Sigma, St. Louis, USA) were used. Differentiation of hMSCs was done using Mesencult osteogenic stimulatory kit(human) (Stem cell technologies,Canada). Fluorescent stains, phalloidin conjugated to Texas Red (Sigma, St. Louis, USA), propidium iodide (PI) and 4, 6-Diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, USA) were used for confocal imaging. Fluorochrome tagged anti-human antibodies, namely CD31-FITC, CD34-FITC, CD-45PE, CD29-FITC, CD44-FITC and CD73-PE for hMSC characterization were all from BD, Biosciences, USA. Primary antibodies of Ki-67 and RUNX2 and FITC conjugated anti-mouse secondary antibody were purchased from Abcam, USA.

3.2 Methods

3.2.1 Intercalation of NC in PCL

Solution intercalation technique was adopted for the fabrication of nanocomposites. Initially, NC was suspended in dichloromethane by stirring for 1h, which was then sonicated in bath sonicator (Misonix Ultrasonic cleaner, USA) for 20 minutes at 40°C to achieve a good dispersion. Similarly, PCL was dissolved in dichloromethane and sonicated in bath sonicator
for 10 minutes at 40°C. Both the solutions were added together and stirred for 1h to ensure proper mixing, followed by sonication at 45°C for 90 minutes. For the purpose of characterization, the solution was then poured into clean glass petri dishes and left covered in a fume hood for 24h in a dichloromethane atmosphere, in order to achieve a slow solvent evaporation. The films were then dried in vacuum oven at 40°C for 24h, to remove residual traces of dichloromethane. Nanocomposite films with a nanoclay content of 1, 2, 4 and 6wt% were thus produced.

### 3.2.2 Fabrication of nano-composite scaffolds

The mixture of chloroform and methanol has been used as a solvent for electrospinning of PCL. PCL (12%w/v) was dissolved in 3:2 (v/v) of chloroform: methanol with constant stirring in room temperature. For electrospinning, nSiO₂(50/100mg) nHAp(32mg) and MF-nHAp(32mg) were mixed with PCL solution and stirred for 1h to obtain homogeneous solution and sonicated for 5 minutes in a bath sonicator (Misonix, USA). For electrospinning NC, the intercalated PCL/NC was dissolved in the solvent by stirring for 1 h. For preparing nanofibrous scaffolds, the solution was taken in a 10ml plastic syringe (BD Discardit II, USA) with a blunt end needle tip and a voltage of 16kV (Gamma High Voltage Research, USA) was applied between the needle tip and the ground collector with a tip-target distance of 12 cm. The flow rate of the solution was optimized at 0.7ml/h for obtaining nanofibers. The target was an aluminium sheet on which the scaffold was electrospun. PCL, PCL/nHAp and PCL/MF-nHAp were the different nanofibrous scaffolds fabricated. The percentage of nHAp and MF-nHAp incorporated were 2.67(w/w).

### 3.2.3 Characterization

#### 3.2.3.1 SEM

The structural morphology of fabricated scaffolds were examined using SEM. Nanocomposite scaffolds fabricated by electrospinning were placed on aluminium stub and coated with gold using sputter coating unit (JEOL JFC 1600, Tokyo, Japan) for 2 minutes at 10mA before imaging. The average fiber diameter was determined by measuring fibers in few areas.
3.2.3.2 TEM
The presence of nSiO$_2$ in the fabricated nanocomposite fibrous scaffolds was examined using transmission electron microscope (TEM) (FEI-Tecnai T-12 TEM, USA). A very thin layer of the electrospun scaffold was taken on the probe and sample was prepared for analysis under TEM. The TEM was operated at 120kV accelerating voltage.

3.2.3.3 Contact Angle Measurement
Contact angle of the fabricated scaffolds were examined using Drop shape analyser (KRUSS-DSA100, Germany). All the samples, PCL, two concentrations of nSiO$_2$ in PCL, different concentrations of NC in PCL, PCL/nHAp and PCL/MF-nHAp, were tested in triplicates. Temporal images of the solution (milli-Q water) droplet on scaffold were automatically taken. From these images, the contact angle values were calculated using the computer simulation software. The chosen experimental conditions were 25°C and about 65% humidity.

3.2.3.4 Mechanical testing of scaffolds
Samples were prepared for analysis in dimensions of 6 cm length and 2 cm breadth. Scaffolds were uniaxially tested to failure at an extension rate of 10mm min$^{-1}$ with ORIENTEC Universal testing Machine STA-1150 RTC (Tokyo, Japan). Four specimens were considered for each electrospun scaffold. The recorded data were used to assess tensile strength with respect to each fiber system.

3.2.4 Leaching studies of gadolinium using inductively coupled plasma-atomic emission spectroscopy (ICP-AES)
Equal weights (7.5mg) of PCL, PCL/nHAp and PCL/MF-nHAp were weighted and incubated in IMDM medium for 1-4 days. The supernatant medium from 1, 2 and 4 day time points were analyzed for the concentration of Gd 3350 using ICP-AES (Thermo Electron IRIS INTREPID II XSP DUO, Thermo Scientific, USA).

3.2.5 Serum protein adsorption studies
Adsorption of serum proteins to nano-composite fibers and PCL fibers on scaffold were evaluated over time. Equal weights (7.5mg) of scaffolds were taken and further cut into small pieces for studies. Scaffolds were placed in 24-well plate, to which media (IMDM with 20%
MSC-FBS) was added and incubated for varying periods (2h, 4h, 6h and 8h) as required. After the incubation of scaffolds in media, they were washed twice with PBS. 400 µl of elution buffer (0.025% sodium dodecyl sulphate & 0.05% CHAPS) (Sigma, USA) was added to every scaffold in order to elute the protein adsorbed from serum in medium. Exception from this elution buffer, only 0.025% sodium dodecyl sulphate was used for NC, nHAp and MF-nHAp containing scaffolds. After 2h incubation in elution buffer, sample and bicinconinic acid (BCA) working reagent were added in a ratio of 1: 8, mixed well and incubated for 30 minutes at 37ºC. A bovine serum albumin solution of 1 mg/mL was made after which, 10 serial dilutions were prepared with elution buffer in order to get a standard curve to obtain the unknown protein values adsorbed on scaffolds. The amount of protein in standards as well as samples were found using BCA assay. Plate was read using a microplate spectrophotometer at 562nm.

3.2.6 Mineralization studies on composite scaffolds
These PCL/nHAp and PCL/MF-nHAp composite scaffolds of 1cm x 1cm x 0.2mm were immersed in SBF solution for a period of 21 days at 37ºC. The SBF was prepared using the method developed by Kukubo et al.16 After soaking for a predetermined time, these scaffolds were rinsed with milli-Q water (Millipore, Billerica, MA), blotted and dried at room temperature. The samples were then characterized by SEM, energy-dispersive spectrometer (EDS) and fourier transform infrared spectroscopy (FTIR) (Perkin–Elmer RX1, USA).

3.2.7 Isolation and characterization of hMSCs from umbilical cord blood (UCB)
UCB was collected in a sterile bottle containing heparin from the hospital after informed consent and approval of the institutional ethics committee. The isolation was done within 2h post delivery. Mononuclear cells were isolated by Ficoll-paque (Stem Cell Technologies, Canada) density gradient separation. These isolated cells were cultured with appropriate media changes until 80% confluence. Cells were subcultured and expanded. Passage 3-6 cells were used for the present study. At the 3rd passage MSCs were characterized by fluorescent activated cell sorter (FACS) analysis. 1x10^5 cells taken in each individual tube were labeled with a specific fluorochrome tagged anti-human antibody. CD31-FITC, CD34-FITC, CD-45PE, CD29-FITC, CD44-FITC and CD73-PE antibody-fluorescent conjugates were analyzed by FACS (FACS Aria, BD Biosciences, USA) using FACS DIVA software (BD Biosciences, USA).
3.2.8 Cell viability of hMSCs on scaffolds

The cell viability assay was done using alamarBlue reagent. Briefly, UCB-hMSCs (Passage 3) were seeded onto nano-composite scaffolds with $10^4$ cells per scaffold. All samples were sterilized by exposing ethylene oxide gas at 40°C for 3h. After 48h incubation, the medium was replaced by 10% alamarBlue in DMEM (Phenol red-). After 8h of incubation, plate was read using microplate spectrophotometer (Biotek PowerWave XS, USA) at 570nm with 600nm set as the reference wavelength. Positive control was PCL nanofibrous scaffold.

3.2.9 Study of silica uptake by hMSCs using flow cytometry

FACS analysis was done on hMSCs incubated for 6h with the nSiO$_2$ particles as well as the PCL/nSiO$_2$ scaffolds. hMSCs (5 X $10^5$ per well) were seeded in medium containing nSiO$_2$ particles or on PCL/nSiO$_2$ scaffolds in a well plate. After incubation, samples were washed four times with PBS to remove the excess silica particles. Cells were trypsinized, pelleted, resuspended and analyzed using FACSARia.

3.2.10 Study of silica uptake by hMSCs using inductively coupled plasma atomic emission spectroscopy (ICP-AES)

hMSCs at a concentration of 5 X $10^5$ per well were seeded in media containing nSiO$_2$ (0.5%/1%w/v) particles or on PCL/nSiO$_2$ (0.5%/1%w/v) scaffolds in a well plate. After 6h of incubation, samples were washed 4 times with PBS to remove the excess silica particles. Cell lysis buffer was added to each well to obtain lysates that were analyzed for silicon content using ICP-AES (Thermo Electron IRIS INTREPID II XSP DUO, Thermo Scientific, USA).

3.2.11 Cell proliferation

Proliferation of the hMSCs on all the scaffolds was performed by alamarBlue assay upon till 6 days, using the standard protocol as mentioned earlier$^{108}$. The proliferation on PCL/MF-HAp scaffolds was further confirmed by MTS assay, DNA quantification by picogreen and Ki-67 staining. MTS assay was performed using the same protocol as alamarBlue. MTS reagent was added after the incubation time and incubated for 2 hours. Plate was read at
For DNA quantification, $10^4$ cells were seeded per scaffold and protocol was followed as per the kit specifications. Ki-67 is a protein expressed in all the dividing cells. Hence it was stained to confirm proliferation on the scaffolds. hMSCs after seeding on the scaffolds were allowed to attach and were serum deprived overnight (≈14h). After which the samples were fixed and permeabilized in 100% methanol for 10 minutes at -20°C. Samples were blocked using 10% anti-goat serum for 1h, treated with the Ki-67 primary antibody overnight at 4°C and washed thrice with PBS. Cells were treated with secondary antibody for 1h and again washed three times in PBS. Cells were mounted with mountant containing nuclear stain (PI).

### 3.2.12 Cell attachment studies

hMSCs ($5 \times 10^3$/scaffold) were seeded on composite scaffolds placed in sterile 24-well tissue culture polystyrene dishes in stationary condition. These scaffolds were sterilized using ethylene oxide gas at 40°C for 3h, prior to seeding cells. Scaffolds with seeded cells were conditioned with IMDM containing 20% MSC-FBS for 12h. After incubation, samples were fixed and analyzed by SEM. Fixing was done with 0.25% gluteraldehyde for an hour at 4°C. The specimens were thoroughly washed and dehydrated using ethanol gradient, gold sputtered in vacuum, and examined using SEM.

### 3.2.13 Differentiation of hMSCs on scaffolds

ALP activity was measured based on the hydrolysis of p-nitrophenyl phosphate liquid substrate to p-nitrophenol. All scaffolds after seeding with hMSCs ($10^4$/scaffold), were cultured in both normal medium and osteogenic medium as required. 50% of medium was replenished every other day. Scaffolds were incubated for time durations of 1, 7, 14, 21 days as required. The cell lysates were prepared by treating with 1% TritonX-100 for 1h. ALP substrate and glycine buffer were taken in microtitre plate to which cell lysate (enzyme) was added with the lysate: substrate ratio being 1:1. The plate was incubated in dark for 30 minutes at 37°C. After incubation, 5M NaOH was added to stop the enzymatic reaction and read using microplate spectrophotometer and measured at 405nm. The ALP activity was determined using the standard graph obtained by serial dilutions of ALP enzyme. Later the values were normalized with the total protein amount (per reaction) measured by the BCA assay using the same protocol as mentioned earlier.
Expression of RUNX2 protein was checked by immunochemistry. The scaffolds were seeded with hMSCs and incubated for 1-21 days as done earlier for ALP studies, in osteogenic medium. These samples at various time points were fixed in 100% methanol for 10 minutes at -20 °C. Then blocked using 1%FBS for 30 minutes, followed by staining with RUNX2 specific primary antibody and incubated overnight at 4 °C. Samples were washed in PBS twice and incubated for 1h with the secondary antibody. They were again washed with PBS twice and counter stained with DAPI to stain the nuclei of cells. Samples were analyzed using confocal microscope (Leica, SP 5 II, Germany).

3.2.14 MRI imaging by T1 weighted contrast of the scaffolds

MR imaging experiments were performed with a 1.5 T clinical scanner (GE Health Care, USA). T1 contrast images were recorded by pulse sequence spin-echo technique with TR/TE ¼ 200 ms/9 ms, FOV 19 by 19 cm², resolution 256 - 256 points, 4 acquisitions and band width 41.67 KHz. The three different electrospun scaffolds PCL, PCL/nHAp and PCL/MF-nHAp were stuck on 18mm round coverslips, and embedded in 2% agar in 12-well plate and the T1 contrast images captured. These three scaffolds were seeded with hMSCs (10⁴cells/scaffold) and incubated in osteogenic medium for 7, 14, 21 and 28 days. At these time points the scaffolds were embedded in 2% agar and analyzed using MRI as mentioned earlier.

3.2.15 Statistical analysis

All quantitative results were obtained from experiments done in triplicates. Data was expressed as the Mean ± SD. Statistical analysis was carried out using one-way ANOVA. A \( p \leq 0.001 \) was considered significant. Student's two-tailed t-test was later performed to find the level of significance between two groups. \( \leq p \leq 0.05 \) was considered to be statistically significant in this case.