Chapter 2
MATERIALS AND METHODS

2.1 In vitro evaluation of electrospun PCL Nano and Micro fibers for hMSCs based bone tissue Engineering

2.1.1 Electrospun PCL scaffolds

PCL scaffolds with nano-fibrous and micro-fibrous structures were fabricated by standard electrospinning technique using an electrospinning setup consisting of a high voltage power supply (Zeonics, India), a syringe pump (KD Scientific, USA) and a custom made stainless steel target.

2.1.1.1 Preparation of PCL solutions and electrospinning setup

PCL solution for micro-fibers and nano-fibers were prepared separately. For nano-fibers, the solution was prepared by dissolving PCL (molecular weight: 43,000–50,000) (Polysciences, USA) in chloroform/methanol (Qualigens, India) mixture (3:1) at a final concentration of 12 wt %. For micro-fibers the solution was prepared by dissolving PCL at a final concentration of 30 wt% in the same solvent. The syringe with a blunt-end needle (Beckton Dickinson, USA) carrying PCL solution was loaded in the syringe pump connected to the positive terminus of the high voltage direct current (DC) power supply. The target was grounded to collect the fibers.

2.1.1.2. Electrospinning of PCL nano-fibers & micro-fibers

Nano-fibers were prepared using 12 wt% PCL solution taken in a 20-mL syringe with blunt-end needle loaded in the electrospinning setup. The polymer solution was electrostatically drawn from the tip of the needle by applying a high voltage between the tip of the needle and the grounded target (collector). The process parameters were optimized for nano-fibers as flow rate - 1mL/h, applied voltage-10 kV, and the needle tip and collector distance (tip-target distance) - 10 cm.
The micro-fibers were spun using 30 wt% PCL solution taken in a 20-mL syringe with a blunt-end needle and the process parameters were optimised for micro-fibers as flow rate- 1mL/h, applied voltage- 8 kV and the tip-target distance 10 cm. In both cases, the electrospun fibers were collected onto aluminium foils placed on the metallic target.

2.1.1.3 Characterization of PCL fibrous scaffolds
Scanning electron microscopy (SEM) images were used to fiber diameter and pore size distribution in each scaffold. An average of the minimum and maximum pore dimensions of at least 25 pores in the electrospun mat was used for the analysis.

2.1.2 Protein adsorption on the fibrous scaffolds
Adsorption of total serum proteins as well as specific adhesive proteins such as fibronectin and vitronectin to nano-fibers and micro-fibers in the scaffolds were evaluated separately at different time periods.

The nano and micro-fibers were electrospun separately to coverslips and were incubated in 24-well plates containing normal media consisting of alpha minimal essential medium (Sigma, USA), 20% fetal bovine serum (Stemcell Technologies, Canada), penicillin–streptomycin 100 units/mL (Invitrogen, USA), and 2mM L-glutamine (Sigma, USA), hereafter referred to as NM, up to respective time points (1, 2, 3, 6, 12, and 24 h for total protein and 1, 3, 6, 12, 24, 48, and 72 h for specific proteins). After incubation period, samples were taken into fresh wells and washed extensively with phosphate buffered saline (PBS).

2.1.2.1. Total protein adsorption
For quantification of adsorbed total serum proteins, adsorbed proteins were eluted from the scaffolds using elution buffer (0.025% sodium dodecyl sulfate, 0.5% 3-[(cholamidopropyl) dimethyl-ammonio]-1-propane sulfonate (CHAPS), and 3mM β-mercaptoethanol (Sigma, USA)) and were quantified using bicinchoninic acid (BCA) assay 1.

2.1.2.2 Specific adhesive protein adsorption
For specific protein quantification, nano-fibers and micro-fibers incubated in NM for specific duration were further incubated for 3 h in blocking solution (5% bovine serum albumin (Sigma, USA) in PBS). Pre-blocked scaffolds were incubated with primary antibodies such as mouse anti-fibronectin and mouse anti-
vitronectin (BioPorto, Denmark) against bovine–fibronectin and bovine-vitronectin overnight at room temperature. The samples were washed twice the following day with PBS and incubated with appropriate goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology, USA). The scaffolds were taken out and the un-conjugated secondary antibodies were washed away with PBS. The scaffolds were then immersed in HRP substrate tetramethyl benzidine liquid substrate system (Sigma, USA) and were incubated for 30 min in dark. The product produced was pipetted into a 96-well plate, and the optical density (OD) was measured at 370 nm using a microplate spectrophotometer (Biotek PowerWave XS, Highland Park, Winooski, VT). A standard graph was prepared using pure bovine fibronectin and vitronectin (Sigma USA), and the adsorbed specific proteins were quantified using the standard graph.

2.1.3 Isolation and primary culture of hMSCs

Bone marrow aspirates were taken from the iliac crest of normal adult donors with the approval of the respective institutional review board and with appropriate informed consent. Aspirates were immediately processed in the cell culture facility for isolation of hMSCs as previously reported in literature. Aspirates were diluted with NM and nucleated cells were isolated with a density gradient (Ficoll-Paque; GE Healthcare, Sweden) and the buffy coat (the white layer located between the Ficoll solution border and the aqueous phase) was collected aseptically with meticulous care and was transferred into a sterile falcon tube. Cells were washed with 25 mL of NM and were centrifuged for 10 min at 3000 rpm. Washing was repeated twice and all the nucleated cells were plated in 20mL medium in a 75-cm² culture flask (BD Falcon, USA) and incubated at 37°C with 5% CO2. After 24 h, non adherent cells were discarded, and adhered cells were thoroughly washed twice with PBS. Cells were cultured and passaged by treating with 0.25% trypsin and 1mM ethylenediaminetetraacetic acid (EDTA) (Invitrogen, USA) for 5 min at 37°C at 80 % confluency.

2.1.4 Characterization of hMSCs

Isolated MSCs were characterized by their tri-lineage differentiation potential and surface marker analysis. Sub-cultured and expanded hMSCs at passages 2-3 were
characterized and used for all experiments.

2.1.4.1 Tri-lineage differentiation potential

The tri-lineage differentiation potential of isolated MSCs was evaluated by their ability to undergo osteogenic, chondrogenic, and adipogenic differentiation.

2.1.4.1.1 Osteogenic differentiation potential

To induce osteogenic differentiation, 5x 10^5 cells were seeded on 25-cm² culture flasks (BD Falcon) and cultured in NM. After the cells reached 90% confluency, the media was changed to osteogenic media. The osteogenic medium was composed of NM with 10mM β-glycerol phosphate (Sigma, USA), 50 µg/mL ascorbic acid-2-phosphate (Sigma, USA) and 10^{-8} M dexamethasone (Sigma, USA), hereafter referred as OM. Medium was changed twice a week. The osteogenic differentiation was confirmed by the mineralization of the matrix at day 28 by staining with alizarin red stain (Sigma, USA) as mentioned in Methods 2.1.7.3.1.

2.1.4.1.2 Chondrogenic differentiation potential

To induce chondrogenic differentiation, approximately 2 x10^5 cells were placed in 15 mL polypropylene tube and centrifuged at 500xg for 5 minutes at room temperature to form a pellet. Then, the pellet was treated with chondrogenic medium. Chondrogenic medium consisted of serum free, high-glucose Dulbecco’s modified Eagle’s medium (DMEM, Gibco BRL) supplemented with 10^{-7} M dexamethasone (Sigma, USA ), 50 µg/ml ascorbate-2-phosphate ( Sigma, USA), 100 µg/mL sodium pyruvate (Sigma, USA), 40 µg/mL L-proline (Sigma,USA), 10 ng/mL transforming growth factor 3 (TGF- 3, Sigma), 500 ng/mL bone morphogenic protein (BMP) 2 and 6 (R&D Systems, USA), and 50 mg/mL ITS+ premix (Becton Dickinson, USA). Medium was changed twice a week. For the evaluation of chondrogenic differentiation, the pellet aggregates were retrieved after 21 days, embedded in paraffin, sectioned to 10 µm sections using an automated microtome (Leica, Germany). The sections were then stained with Safranin-O (Sigma, USA) and Toludine blue (Sigma USA) and were mounted on microscopic slides and histological evaluation was performed.

2.1.4.1.3 Adipogenic differentiation potential

Cells were treated with adipogenic medium, to induce adipogenic
differentiation. Adipogenic medium consisted of NM supplemented with 0.5 µM Isobutylmethylxanthine (IBMX), 50 µM indomethacin (IM) and 0.5 µM dexamethasone. Media change was given twice every week. Adipogenesis were evaluated by Oil-red O (Sigma, Sigma) staining at 21 days.

2.1.4.2 Surface Marker Analysis

The isolated hMSCs were cultured in NM up to passage 3 prior to analysis for surface markers. The cells were labelled with the following fluorochrome-conjugated mouse anti-human antibodies: CD31-FITC, CD34-FITC, CD44-FITC, CD45-PE, CD73-PE (BD Biosciences, USA), Stro-1 (Invitrogen, USA), and goat anti mouse-FITC (Sigma USA). At least 50,000 events were acquired and analyzed using a fluorescent-activated cell sorting system (FACS-Aria, BDBiosciences, USA) and quantified using FACS DIVA software.

2.1.5 hMSCs on PCL scaffolds

Isolated hMSCs were seeded to pre-sterilized and pre-conditioned nano- or micro-fibrous scaffolds under aseptic conditions.

2.1.5.1 Single seeding and dual seeding

Two different seeding strategies (single and dual seeding) were employed for seeding hMSCs to the electrospun scaffolds. A custom designed electrospun scaffold holder (Figure 2.1) was used to hold the electrospun wafers intact in the culturing media while seeding the cells. For single seeding, electrospun scaffolds pre-incubated in NM for 6 h were aseptically placed in the scaffold holder and were seeded with hMSCs on the upward facing surface. In the case of dual seeding hMSCs were seeded on both sides of the electrospun wafer. For that, after the initial seeding, cells were incubated for 4 h and the scaffold along with the holder was flipped with the electrospun wafers with the help of sterile forceps. After that the second side of the wafer was seeded.
2.1.5.2 Attachment and spreading of hMSCs on nano-fibers

Nano-fibrous scaffolds were pre-incubated in NM for 6 h for protein adsorption. The pre-incubated scaffolds loaded in the scaffold holder were placed in 24-well plates and were seeded with hMSCs, at a seeding density of $2 \times 10^4$ cells/scaffold. The adhesion and spreading behaviour of hMSCs on the nano-fibrous scaffolds over time were evaluated by fixing the cells at 2, 4, 6, 8, 12, and 24 h of incubation. For that, cell seeded nano-fibrous scaffolds were carefully taken out, washed in PBS and fixed with 2.5% glutaraldehyde in PBS at room temperature for 1 h. The specimens were thoroughly washed with PBS and dehydrated using ethanol gradient, air dried, platinum sputtered in vacuum (JSM-6490 LA, Jeol, Japan), and examined using scanning electron microscope (JSM-6490LA, Jeol).

2.1.5.3 Attachment spreading of hMSCs on micro-fibers

Micro-fibrous scaffolds were pre-incubated in NM for 6 h for protein adsorption and were seeded with hMSCs similarly as done for nano-fibers. The cell attachment and spreading of hMSCs on nano-fibrous scaffolds were evaluated and compared with micro-fibrous scaffolds by fixing the cells after 12 h of incubation.

2.1.5.4 hMSC infiltration on nano-fibers & micro-fibers

Infiltration of hMSCs to the nano and micro-fibrous scaffolds was evaluated by cell infiltration assay and confocal microscopy. For the assays, fibrous scaffolds with approximately 200 µm thickness were placed in custom designed scaffold holders (Figure 2.1). Then the scaffolds were seeded with hMSCs ($5 \times 10^5$ /scaffold) and were incubated for 24 h at 37°C. After 24 h, scaffolds were removed from the scaffold holder and the total numbers of cells present in the
wells were counted after trypsinization. The total number of cells present in the wells is an indirect measure of the cells retained in the scaffolds. Scaffolds with hMSCs were removed and stained with F-actin (Invitrogen, USA) and optical slicing of the cells seeded scaffolds were performed in the confocal microscope. Sliced images were reconstructed as 3D image using the Z – stacking facility of the confocal microscopy, to visualize the cell infiltration.

2.1.6 hMSC proliferation on PCL nano-fibrous scaffolds
The proliferation of hMSCs on scaffolds with nano-fibrous structure was assessed for 7, 14, 21, and 28 days using Alamar Blue assay \(^3, \text{4}\). The Alamar Blue assay incorporates an oxidation/reduction indicator that both fluoresces and changes colour in response to chemical reduction of growth medium resulting from cell proliferation/metabolic activity. Briefly, scaffolds in coverslips were sterilized by alcohol/UV treatment and were placed in a 24-well plate. The nano-fibrous scaffolds were pre-incubated with serum containing media for 6 h. The cells were seeded at a concentration of 1x10\(^4\) cells/well on a 24-well plate on PCL nano-fibrous scaffolds (1 cm\(^2\)) fixed to the cover slips. At the end of the incubation period, scaffolds were placed in fresh wells in phenol red-free serum-free medium, and Alamar Blue was added to the wells. The nano-fibrous scaffolds containing hMSCs were incubated overnight in Alamar Blue-containing medium. Thereafter, the media were pipetted into 96-well plates, and the OD was recorded using a microplate spectrophotometer at 570 nm, with 600nm set as the reference wavelength. A standard graph was prepared by seeding different number of cells in 24-well plates, and the OD was evaluated. The cell numbers at different days were approximated from the standard graph.

2.1.7 Osteogenic differentiation of hMSCs on nano-fibrous scaffolds
All assays were carried out on second or third-passage hMSCs. One day after seeding the cells (at a concentration of 1x10\(^5\) cells/scaffold) on 24-well plates, and NM was exchanged with OM. BMP-2 (25 ng/mL) and 10\(^\text{-8}\)M Parathyroid hormone (PTH) were used separately in NM and OM for evaluating the response of hMSCs on scaffolds having nano-fibrous structure to these regulators of osteogenic differentiation. A similar experiment was also carried out
with the addition of the inhibitor cyclohexamide at 1–2 h prior to the addition of OM, BMP-2, or PTH.

2.1.7.1 Alkaline Phosphatase Activity

The alkaline phosphatase (ALP) activity of hMSCs on nano-fibrous scaffolds was determined quantitatively. For this, ALP activity at 1, 7, 14, and 21 days after treatment was measured based on the hydrolysis of p-nitrophenyl phosphate liquid substrate (Sigma, USA) to p-nitrophenol. The cell lysate from the nano-fibrous scaffolds was prepared by treating with 1% Triton X-100 for 50 min and sonication for 10 min. ALP substrate was taken in a microtiter plate, and cell lysate was added to the substrate (1:1). After 30 min, 1M sodium hydroxide (Sigma, USA) was added to stop the enzymatic reaction. The OD of the reaction product was measured at 405nm in the microplate spectrophotometer. The ALP activity in the cell lysate was determined from the standard graph and was normalized to the total protein amount measured using the BCA assay.

2.1.7.2 Real time PCR

The hMSCs were grown on nano-fibrous scaffolds for 2 weeks under normal and osteogenic differentiation inducing conditions such as OM, NMB (NM+BMP-2), OMB (OM+BMP-2), NMP (NM+PTH), and OMP (OM+PTH). Complimentary DNA (cDNA) from cells grown on scaffolds was prepared directly on the 1st, 7th, and 14th day using Fastlane Cell cDNA kit (Qiagen, GmbH, Hildon, Germany), according to the manufacturer’s instructions. Three separate scaffolds were selected for each condition, and cDNA was synthesized separately. For quantitative determination, synthesized cDNA was pooled, and analysis was done in duplicates using Real-Time PCR system 7300 (Applied Biosciences, Foster City, CA). All reactions were carried out in a total volume of 20 µL, containing 10 µL Power SYBR Green Master mix (Applied Biosystems, Foster City, CA), 2 µL QuantiTect Primer assay (Qiagen), 1 µL cDNA, and 7 µL of RNAse-free water at an annealing temperature of 55°C for 40 cycles. Primers that were used are listed in Table 2.1. For each gene, the quality and specificity were assessed by examining polymerase chain reaction (PCR) melt curves after real-time PCR. The results were normalized by expressing them relative to the amount of glyceraldehyde-3-phosphate dehydrogenase mRNA determined in each
sample. The results of real-time PCR were represented as fold increase with respect to non-induced and induced hMSCs at 24 h.

2.1.7.3 Mineralization
The mineralization of hMSCs on the nano-fibrous scaffolds was analyzed and quantified using an alizarin red-based assay.

2.1.7.3.1 Alizarin red based assays
The cells grown on the nano-fibrous scaffolds were washed with PBS and fixed with paraformaldehyde for 15 min. Then the cell-seeded constructs were extensively washed with distilled water prior to the addition of 500 mL of 40mM alizarin red stain (pH 4.1). The plates were incubated at room temperature for 20 min with gentle shaking. After aspiration of the unincorporated dye, the wells were washed four times with distilled water while shaking for 5 min. The plates were then left at an angle for 2 min to facilitate removal of excess water, reaspirated, and then stored at -20°C before dye extraction. For quantification of mineralization, alizarin red stain was extracted from the scaffold in 500 µL of cetylpyridinium chloride for 1 h. Then 200 µL of the extract was taken in a microtiter plate, and the absorbance was measured in a microplate spectrophotometer (Biotek PowerWave XS) at 550 nm. The scaffolds without hMSCs were treated similarly as that done for scaffolds with hMSCs in OM to check the mineral deposition from OM onto the scaffolds.

2.1.7.3.2 SEM analysis
For SEM analysis, the hMSC seeded PCL nano-fibrous scaffolds were thoroughly washed with PBS, fixed and dehydrated through alcohol gradient, sputter coated with platinum, and attached to the sample holder with a conductive carbon tape.

2.1.7.3 Energy dispersive analysis by X-ray (EDAX)
EDAX microanalysis was employed to detect the presence of calcium (Ca) and phosphorous (P) in the sample. The maps of the relative distribution of these elements were acquired at 8 kV, with an acquisition time of 10 min. The quantity of Ca and P in each sample was measured, and the Ca:P ratio was calculated.

2.1.8 Statistical analysis
All quantitative results were obtained from triplicate samples. Data were
expressed as the mean±standard deviation. Statistical analysis was carried out using SPSS software (Version 11, licenced to Amrita Viswavidyapeetham). Single-factor analysis of variance was used to test the equality of the means. Post hoc test (Tukey honestly significant difference [HSD]) was used for evaluation of significant difference within groups. A value of p<0.05 was considered to be statistically significant.

2.2 Gelatin nanoparticles incorporated semi-synthetic PCL nano-fibrous scaffolds for bone tissue engineering

2.2.1 Preparation of gelatin nanoparticles (nG)

Gelatin nanoparticles were prepared using a modified two-step desolvation method. For the preparation, 1.25 g of gelatin (type A from porcine skin, Sigma, USA) was dissolved in 25 ml of distilled water and was heated gently to 50 °C. pH of the solution was adjusted to 3.6–3.7 using 0.1N hydrochloric acid (HCl) (Merck, India) and 25 ml of acetone (Merck, India) was rapidly added to the solution. A white precipitate was formed soon after the addition of acetone. The precipitate was collected and re-dissolved in 25 ml of deionized water and the pH was maintained at 3.6-3.7 using 0.1N HCl. Then the solution was kept with constant stirring at 40 °C and 75 ml of acetone was added at a constant speed (5 ml/min). A milky white solution was obtained when the added volume of acetone reached 55–56 ml. Soon after the formation, nanoparticles were cross linked by adding 0.2 ml of 25% glutaraldehyde (Fluka, USA) and were stirred continuously for 1 h followed by overnight incubation at room temperature without stirring. After incubation, the nanoparticle suspension was neutralized by adjusting the pH to 7.4 using 5 M NaOH. The neutralized nanoparticle suspension was pelleted at 17,000 rpm for 20 min in a refrigerated centrifuge and resuspended in methanol (Merck, India). This resuspended nanoparticle suspension was aliquoted quantified and stored at 4°C for further analysis.

2.2.2 Characterization of prepared nG.

Prepared gelatin nanoparticles were characterized using SEM, AFM and DLS.

2.2.2.1 SEM analysis

SEM was employed to study the morphology as well as particle size of nGs. Prepared nGs were diluted in methanol, dropped into aluminium stubs and
allowed to air dry. Dried samples were platinum sputtered using the sputter coating instrument and were imaged under SEM.

2.2.2.2 Atomic Force Microscopy (AFM) analysis

AFM was also used to study the morphology as well as particle size of nGs. Nanoparticles were diluted the same way as that of the sample preparation for SEM and were dropped on to mica sheets and was air dried. Air dried samples were images using the AFM.

2.2.2.3 Dynamic Light Scattering (DLS) analysis

The mean particle size and size distribution of prepared nGs were analyzed using dynamic light scattering measurements (DLS-ZP/particle Sizer Nicomp 380 ZLS, particle sizing system).

2.2.3 Quantification of nG

Standard BCA assay was used to quantify the gelatin content in the nanoparticle suspension. For the assay, neutralized nanoparticles and BCA reagent (bicinchonic acid: cupric sulphate in the ratio 50:1, Sigma, USA) were added in a ratio of 1:8 and mixed well, and incubated for 30 min at 37 °C in the dark. The product produced was pipetted into a 96-well plate, and the optical density (OD) was measured at 370nm using a microplate spectrophotometer (Biotek PowerWave XS, USA). A standard graph was prepared using pristine gelatin solution (Sigma, USA), and the amount of gelatin in the nanoparticle suspension was quantified.

2.2.4 Electrospinning of PCL and PCL_nG nano-fibers

Nano-fibrous scaffolds were fabricated by slightly modifying our previous experimental (2.1.1). Electrospinning was performed by dissolving PCL (molecular weight: 43,000–50,000, Polysciences, USA) in a chloroform/methanol mixture (3:1) (Merck, India) at a final concentration of 16 wt%. The polymer solution was taken in a 20 ml syringe with a blunt-end needle and loaded in the electrospinning setup. The polymer solution was electrostatically drawn from the tip of the needle by applying a high voltage between the tip of the needle and the grounded target (collector) using high-voltage power supply (Gamma High Voltage, USA). The flow rate of the solution was kept at 1 mL /h, the applied voltage at 10 kV, and the needle tip and collector distance (air gap) at 10 cm.
PCL_nG scaffolds were electrospun using standardized electrospinning conditions as above. Briefly, nanoparticles were thawed at room temperature for 30 min. The nanoparticle suspension was sonicated (Sonics-Vibra cell, USA) and the particle size was re-confirmed by DLS and BCA assay, respectively. PCL solution in chloroform/methanol mixture was prepared and nanoparticles were added to the PCL solution at different concentrations (5-15 wt % of PCL) under constant stirring. The PCL_nG mix was continuously stirred for 1 h and was electrospun with the same spinning parameters as used for PCL solution. PCL_nG scaffolds containing 15 wt% of nGs were used for comparing the physical, chemical and biological properties with PCL scaffolds.

2.2.5 Characterization of the scaffolds

The physico-chemical characterization of the electrospun scaffolds were performed using SEM, FTIR, TG/DTA and contact angle analysis

2.2.5.1 SEM analysis.

The morphology of nano-fibrous scaffolds was studied by SEM. To improve the sample conductivity, scaffolds were coated with gold, using a sputter coater. The diameter of the fiber was measured manually from SEM micrographs using the text/multi-point measurement tool in the SEM.

2.2.5.2 Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) was used to identify the infrared absorption peaks of gelatin to confirm its presence in the composite. The spectral scan was carried out from 4000 to 400 cm$^{-1}$ using an IR-spectrometer (Perkin Elmer Spectrum RX-1, USA).

2.2.5.3 Thermogravimetric (TG) and Differential Thermal Analysis (DTA)

TG/DTA analysis of the scaffolds was carried out using a TG/DTA instrument (XSTAR, TG-DTA 6200, USA) at a range of 25–500 °C.

2.2.5.4 Contact angle analysis

Wettability of the scaffolds was measured using a water contact angle system supported by videocam equipment (KRUSS, DSA 100, Germany). Continuous recording of contact angles was done from time zero and stable contact angle values were obtained, in order to eliminate any influence of subsequent perfusion flow through the nano-fibrous scaffolds.
2.2.6 *In vitro* degradation of PCL and PCL_nG scaffolds

The influence of incorporation of nGs on the degradation behavior of the scaffolds was analyzed both qualitatively and quantitatively. Samples were incubated in simulated body fluid (SBF) at 37 °C with shaking for a period of three months. SEM analysis was carried out to evaluate the morphological changes at different time points viz., 2, 4, 6, 8 and 12 weeks. After three months, samples were collected by draining SBF through a 40 µm cell strainer (BD, USA), kept on blotting paper for 5 min and vacuum dried for seven days. Dried samples were weighed on an electronic balance and the mass retained (in percentage) of each scaffold was calculated by

\[
\text{Dry weight retained} = \frac{D_{Mt}}{D_{Mi}} \times 100
\]

Where \(D_{Mt}\) is the dry weight of the scaffold after three months incubation in SBF and \(D_{Mi}\) is the initial dry weight of the sample before incubation in SBF.

2.2.7 hMSCs on PCL_nG nano-fibrous scaffolds

All cytocompatibility experiments performed using hMSCs. Isolation characterization and culturing of hMSCs were performed as previously described in 2.1.3 and 2.1.4. Scaffolds were sterilized using an ethylene oxide sterilizer (Sun Health Care, India). In all experiments, before cell seeding, scaffolds were conditioned by incubating with 10% FBS at 37 °C for 6h.

2.2.8 Attachment and spreading of hMSCs on PCL_nG nano-fibrous scaffolds

The adhesion and spreading of hMSCs on the developed scaffolds was evaluated using SEM and confocal microscopy. hMSCs were seeded on the nano-fibrous scaffolds at a concentration of \(5 \times 10^4\) cells/scaffolds and incubated under standard cell culture conditions. For evaluating hMSC adhesion, after 3 h of incubation, the cell-seeded nano-fibrous scaffolds were carefully taken out, washed in PBS and fixed with 2.5% gluteraldehyde in PBS at room temperature for 1 h. The specimens were thoroughly washed with PBS and dehydrated using ethanol gradient, air dried, platinum sputtered and examined using SEM. For evaluating the spreading behavior of hMSCs, the cell-seeded nano-fibrous scaffolds were carefully taken out, washed in PBS and fixed with 4% paraformaldehyde (Sigma, USA) in PBS at room temperature for 15 min. The fixed cells were permeabilized with 0.1% triton (Sigma, USA) and were
immunostained against F-actin using FITC-conjugated phalloidin (Invitrogen, USA) for 1 h at room temperature. After 1 h, specimens were washed with PBS and were analyzed in the confocal microscope.

2.2.9 Viability of hMSCs on PCL_nG nano-fibrous scaffolds

Both qualitative and quantitative determinations of the viability of hMSCs on the developed scaffolds were performed. For quantitative determination of cell viability, scaffolds were taken in 24-well plates and were seeded with $5 \times 10^4$ cells/scaffold and standard alamar blue assay was performed after 24 and 48 h. Qualitative analysis of the cell-seeded scaffolds after seven days was performed using a live/dead cell viability assay kit (Molecular Probes, USA) according to the manufacturer’s instructions. It was then washed with PBS and imaged using confocal laser scanning microscopy (Leica, TCS SP5 II, Germany).

2.2.10 Proliferation of hMSCs on PCL_nG nano-fibrous scaffolds

The proliferation of hMSCs on the developed scaffolds was assessed for one, three, five and seven days by quantifying the DNA content using picogreen assay. Scaffolds were placed in 24-well plates and were seeded with $10^4$ cells/scaffold. After respective time points, cells were lysed using celLytic (Sigma, USA). The DNA content in the lysate was quantified using pico green assay as per manufacturer’s protocol and the intensity of fluorescence was measured with a multimode detector (DTX 880, Beckman Coulter, USA) at excitation and emission wavelengths of 485 and 535 nm, respectively. Relative fluorescence units were correlated with cell number using a calibration line constructed with increasing concentrations of cells.

2.2.11 Osteogenic differentiation of hMSCs on PCL_nG nano-fibrous scaffolds

For differentiation studies, hMSCs were seeded on scaffolds ($2.5 \times 10^4$ cells/scaffolds) and maintained in NM for three days under static conditions. After three days, cells were induced to differentiate into the osteogenic lineage by providing the OM. Thereafter cells were subjected to media change twice a week. Differentiation of hMSCs on the developed scaffolds was evaluated by alkaline phosphatase activity (ALP), expression of osteocalcin and bone sialoprotein, and matrix mineralization.

2.2.11.1 Alkaline phosphatase activity
ALP activity was determined quantitatively at 1, 7, 14 and 21 days after treatment based on the hydrolysis of p-nitrophenyl phosphate liquid substrate as previously described in section 2.1.7.1

2.2.11.2 Electrophoresis and western blot analysis
Detection of osteogenic differentiation markers on hMSCs growing on the nanofibrous scaffolds was done by electrophoresis and western blot analyses. MSCs were seeded on the scaffolds at a concentration of $2.5 \times 10^4$ cells/scaffold. After 72 h, normal media were replaced by differentiation media. At day 7 and day 14, cells were lysed in modified lysis buffer (CelLytic containing protease inhibitors). The lysates were cleared by centrifugation at 15,000 rpm for 10 min at 4° C. The total protein concentration of the resulting supernatant was determined using BCA assay. Equal amounts of proteins were separated by gel electrophoresis under reducing conditions. The separated proteins were then electrophoretically transferred to a PVDF membrane by applying 30 mV overnight at 4° C. The resulting PVDF membrane was then washed twice with PBS containing 0.1% Tween 20 (PBS-T) and subsequently blocked overnight with PBS-T containing 5% non-fat dry milk (Sigma, USA). Membranes were then incubated overnight at 4° C with one of the following primary antibodies (Abcam, USA) at the indicated dilutions: anti-osteocalcin (1:2000), anti-bone sialoprotein (1:2000) and anti-GAPDH (1:5000). Blots were then washed 3–4 times with PBS-T and incubated with the appropriate HRP-conjugated secondary antibody (Abcam USA) for more than 1 h. After the incubation period, the membranes were washed 3–4 times with PBS-T, incubated with an enhanced chemiluminescent substrate (Millipore, USA) and then exposed to x-ray film.

2.2.11.3 Mineralization of the scaffolds
The mineralization of hMSCs on developed scaffolds was analyzed and quantified using alizarin red staining and its semi-quantitative assay.

2.2.11.3.1 Alizarin red staining
Alizarin red staining and its semi-quantitative assay were performed as previously described in section 2.1.7.3.1.

2.2.12 Statistical analysis
All results were obtained from triplicate samples. Quantitative results are represented as mean± standard deviation. Student’s t-test was performed to determine statistical significance in results. p value of less than 0.05 (p < 0.05) was considered to be statistically significant.

2.3 Development and in vivo evaluation of 3D bioengineered constructs with spatially separated osteogenic and angiogenic zones

2.3.1 Synthesis of Hydroxyapatite (HAₚ)
HAₚ microparticles were prepared by a precipitation route by slightly modifying the previously reported protocol⁹. Briefly, 37.04 g of Ca(OH)₂ was added to 1000 ml distilled water and stirred at 100°C for 2 h. 0.3M of H₃PO₄ solution was added drop wise to the above at a rate of ~ 2mL/min. pH of the solution was continuously monitored and the addition was stopped when the pH reached 7.4. The reaction was allowed to proceed at 100°C for another 4 h, and then kept overnight at ambient temperature. The following day, supernatant was discarded and the precipitate was washed four times with distilled water with subsequent centrifugation. The washed precipitate was kept in the freezer overnight and subsequently dried under vacuum at 60°C.

2.3.2 Development and characterization of macroporous HAₚ discs
HAₚ discs were prepared by dry compaction method and naphthalene (Sigma, USA) was used as a porogen in the disc. For the preparation, HAₚ particles were mixed with naphthalene at 1:1 ratio in a custom designed dye having a bore size of 8 mm diameter (Figure 2.3) and compressed using a manual hydraulic press (Kimaya engineers, India) to make 8 mm diameter ceramic discs having 400 µm thickness. Prepared discs were sintered in a muffle furnace (High Heat, India) at 1000°C for 1 hr. Sintered ceramic discs were characterized for its crystallinity using X-ray diffractometry [Rigaku Dmax-C fitted with Cu-Kα (λ = 1.541 Å)]. The morphology and pore distribution were evaluated using SEM. Spectroscopic evaluation of HAₚ discs was carried out using FTIR (Perkin-Elmer Spectrum RX1)
2.3.3 Development of PCL_nG nano and micro-fibrous scaffolds

PCL nano-fibrous scaffolds were prepared as mentioned in section 2.2.4. For micro-fibers 30 wt% PCL_nG solution was prepared and electrospun using the same electrospinning parameters as mentioned in 2.2.4.

2.3.4 Isolation of HUVECs from umbilical cord

HUVECs were isolated from human umbilical cord by slightly modifying the previously reported protocol. Umbilical cord was collected from the gynaecology department of Amrita Institute of Medical Sciences with the approval of the respective institutional review board and with appropriate informed patient consent. The outer surface of the collected cord (approximate length 20 cm) was cleaned with PBS. At one end of the umbilical vein in the cord, a feeding needle was carefully inserted without damaging the surrounding tissue and it was properly held in place using a clamping scissor. The needle was connected with a 20 mL syringe filled with 20 mL PBS containing 2 mM EDTA. The vein was rinsed using this PBS until no remnants of blood are visible in the elute. After proper rinsing, the syringe was replaced with a second syringe filled with 5 mL of pre-warmed 0.25 % trypsin-EDTA. The other end of the vein was closed by connecting to a needle with end-cap clamping with a clamping scissor. To the vein, 5 mL of the pre-warmed trypsin-EDTA solution was injected. Then the cord was suspended in U-shape in a beaker containing 500 ml of pre-warmed sterile distilled water and was incubated in 37°C for 15-20 minutes. After incubation the cord was placed on soft sterile cotton and was massaged aseptically in the hood for 5 minutes with two fingers. Another syringe with 8 mL of PBS

Figure 2.3.1: Custom designed dye for developing HAp discs. A is the top and bottom portions of the dye and B is the accessory components used for making the ceramic disc using the dye.
containing 2 mM EDTA and was connected to the feeding needle. The needle end cap from the other end of the cord was removed and the cord was flushed with PBS containing 2 mM EDTA to a 50 mL falcon tube containing 2 mL FBS. The elute was centrifuged at 200 x g and the supernatant was carefully aspirated. The pellet was resuspended in 15 ml endothelial growth media EBM-2 (Lonza, USA) and cells were directly plated in gelatin coated T75 flasks. Unattached cells were removed after 6 hrs and the attached cells were passaged at 80 % confluency.

2.3.4.1 Characterization of HUVECs

HUVECs at passage 2-3 were characterized using immune cytochemistry, LDL uptake and nitric oxide release.

2.3.4.1.1 Immunocytochemistry

HUVECs were seeded onto the gelatin coated cover slips and were incubated in EGM -2 growth media. After 24 h, cells were fixed with 4% paraformaldehyde and were blocked with 1% BSA in PBS for 30 min to minimize unspecific binding of the antibodies. Blocked cells were incubated with the diluted (1:1000) FITC conjugated mouse anti-human CD-31 (Sigma, USA) antibody in 1% BSA in PBS in a humidified chamber for 1 hr at room temperature. After incubation, cells were washed 3 times with PBS and were mounted using Vectashield (Vector Labs, USA). Mounted cover slips were sealed with sealants to prevent drying and movement under microscope and were evaluated using confocal microscopy.

2.3.4.1.2 Acetylated low density lipoprotein (AcLDL) uptake

AcLDL uptake by HUVECS were evaluated by visualizing the uptake using Dil conjugated AcLDL (Invitrogen USA). For the assay, HUVECs were seeded on gelatin coated cover slips and cells were incubated with 10 µg/mL DiI-Ac-LDL (Invitrogen, USA) at 37°C in serum free EGM media for 4 h. After 4h the media was removed and the cells were washed twice with PBS, and then fixed with 4 % paraformaldehyde. Coverslips were mounted as mentioned above (2.3.4.1.1) and were visualized using confocal microscopy.

2.3.5 Isolation and culturing of hMSCs

hMSCs were isolated and expanded as described in section 2.1.3 and characterized as described in 2.1.4. Passage 2-3 cells were used for all experiments.
2.3.6 Pre-differentiation of hMSCs to pericytes and characterization

hMSCs were pre-differentiated to pericytes using TGF–β treatment as previously reported\textsuperscript{11}. Passage 2 cells were seeded in T75 flasks in NM containing 1ng/mL TGF-β for 5 days. After 5 days cells were characterized based on the smooth muscle cell specific marker expression. For characterization cells were fixed with 4% paraformaldehyde in PBS. Thereafter, as per the manufacturer’s protocol, the cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min. Permiabilized cells were blocked with 1% BSA in PBS for 15 min to limit the nonspecific binding sites. Blocked specimens were incubated with primary antibodies containing smooth muscle α-actin (Sigma, USA) and calponin (Sigma, USA) for 1 h at room temperature. After 1h unbound antibodies were removed by washing in PBS twice and were incubated with flurochrome labelled anti-mouse IgG secondary antibodies such as Alexa fluor 488 and Texas red (Invitrogen, USA) respectively for 1 h. After incubation unbound secondary antibodies were washed away using PBS and samples were mounted as mentioned in 2.3.4.1.1 and were visualized using confocal microscope.

2.3.7 Preparation of collagen/fibronectin gel containing HUVECs and pericytes

Collagen/fibronectin gel containing HUVECs and pericytes was prepared as previously reported\textsuperscript{12}. Collagen solution (Advanced BioMatrix, USA) containing 90 µg/ml fibronectin (Advanced Biomatrix, USA) and 25 mM HEPES (Sigma, USA) was prepared at 4\textdegree C and was mixed with 10X RPMI in 9:1 ratio. pH of the solution was adjusted by using 1 N NaOH (Sigma, USA). To this solution, 1x 10\textsuperscript{6} HUVECS and 2.5 x 10\textsuperscript{5} hMSCs pre-differentiated into pericytes were added per mL. This suspension was pipetted into appropriate well plates and warmed to 37\textdegree C for 30 min to allow polymerization of collagen. Soon after gel formation the gel was covered by carefully adding pre-warmed EGM-2 media and were kept in the CO\textsubscript{2} incubator.

2.3.7.1 Characterization of the vascular network in the gel

In vitro vascular network formation in the gel was evaluated using an inverted phase contrast microscopy (Leica, DMIL2, Germany). HUVECs and pericytes containing Collagen/fibronectin solution was placed in cover glass kept in 6 mm
cell culture dishes. After gel formation, EGM media was added to the dish without disturbing the gel. Then the coverslips with the gel was kept in CO₂ incubator and the network formation was evaluated 1, 3 and 5 days after gel formation.

2.3.8 Fabrication of angiogenic zones in micro-fibrous polymeric platforms
Prepared PCL-nG micro-fibrous scaffolds (dia 8mm) were sterilized using ethylene oxide sterilizer (PCI LtD, India). Pre-sterilized scaffolds were placed in the custom designed scaffold holders (Figure 2.1) placed in 12 well plates. HUVECs and pericytes containing collagen/fibronectin solution was prepared as mentioned above (2.3.8) and 100 µl of the gel solution was added into the micro-fibrous scaffolds at 4°C. The scaffolds with gel solutions were carefully placed in the incubator and allowed the gel formation for 30 minutes. After the gel formation on the scaffolds, sufficient EGM media to cover the formed gel was added in the wells without disrupting the gel.

2.3.9 Fabrication of angiogenic zones in microporous hydroxyapatite ceramic platforms
Prepared macroporous hydroxyapatite discs (8 mm) were sterilized using the ethylene oxide sterilizer. Pre-sterilized ceramic discs were placed on the custom designed scaffold holder in 12-well plates. The gel solution was prepared the same way as that mentioned above (2.3.7) and 100 µl of the gel solution was added into the macroporous ceramic scaffolds at 4°C and treated the same way as mentioned above.

2.3.10 In vitro evaluation of the microvascular network formation on the angiogenic zone
In vitro vascular network formation in the prepared angiogenic zones were evaluated using pre-labeled HUVECs and Pericytes as well as by calcein staining.

2.3.10.1 Live cell imaging using pre-labelled HUVECs and pericytes
Both HUVECS and pericytes were pre-labelled using lipophilic dyes PKH 26 and PKH67 (Sigma, USA) as per manufactures’ protocol. HUVECs were labelled with red dye (PKH 26) and pericytes were labelled with green dye (PKH67). For labelling, 1mL cell suspension containing 2 x 10⁷ HUVECs or Pericytes were taken in a 15 ml tube and were washed with serum-free media. Then cells were centrifuged at 400 x g to get a loose pellet. The supernatant was carefully
aspirated completely without disturbing the pellet. 1 mL of diluent C is added to the pellet and was resuspended with gentle tituration. Immediately prior to staining, a 2X Dye solution was prepared (green dye for pericytes and red dye for HUVECs) in diluent C by adding appropriate amount of the dye to the diluent solution. 1 mL of 2X dye solution was rapidly added to 1 mL of 2X Cell Suspension and was mixed by gentle tituration. The cell/dye suspension was incubated for 5 minutes after which, 2 mL of serum was added to the solution to stop the reaction. The cells were centrifuged at 400x g for 10 minutes at 20-25 °C and carefully remove the supernatant. The cells were resuspended in 10 mL of complete media and was transferred to a fresh 15 mL tube and was centrifuged at 400 x g for 5 minutes at 20-25 °C. This procedure was repeated thrice. After the final wash, cells were suspended in 10 mL of complete media and the required number of cells for angiogenic zone preparation was added to the collagen/fibronectin gel solution in both micro-fibrous polymeric platform and microporous ceramic platform. Prepared angiogenic zones were incubated in endothelial growth media for 1 day and were evaluated for vascular network formation using live cell imaging facility in the confocal microscope.

2.3.10.2 Live cell imaging after calcein staining

Angiogenic zones were prepared on the micro-fibrous polymeric and macroporous ceramic platforms as mentioned in section (2.3.10 and 2.3.11). Angiogenic zones were incubated in the complete endothelial media (EGM-2) for 48 h. After 48 h the cells in the angiogenic zones were stained with calcein (Invitrogen, India). Briefly, 2 µM calcein solution was prepared in sterile PBS from the 4 mM stock. The angiogenic zones were taken out from the scaffold holders and were placed in confocal microscopic dishes (ibdi, USA) containing 200 µl of prepared calcein solution. Angiogenic zones were incubated in the calcein solution for 30-45 minutes and were imaged in the live cell imaging facility of the confocal microscope.

2.3.11 Fabrication of nano-fibrous osteogenic zones and its evaluation

Nano-fibrous osteogenic zones were engineered by dual seeding of hMSCs on the PCL_nG scaffolds as described in section 2.1.5.1. Scaffolds having 8 mm diameter (100 µm thickness) were punched using biopsy punches and were placed
in a custom designed scaffold holder. Scaffold holders along with scaffolds were kept in 12 well plates. hMSCs (passage 2-3) differentiated to osteogenic lineage by growing them in OM for 1 week were seeded on the scaffolds (1x10^5 cells/side). After 4 hrs, the scaffolds were flipped along with the scaffold holders and the seeding was repeated to make sure cells on both surfaces of the nano-fibrous wafer. Seeded nano-fibrous discs were cryosectioned (Leica, CM1510S3, Germany) and stained with DAPI (Sigma, USA) and imaged using a florescent microscope (Olympus, BX51 with camera DP71) in order to visualize cells on both sides of the nano-fibers. The nano-fibrous scaffolds seeded with osteogenic differentiation induced hMSCs are hereafter mentioned as osteogenic zones.

2.3.12 Development of 3D layered constructs

3D layered constructs were engineered by aseptically layering the bio-engineered angiogenic (in ceramic platform or micro-fibrous platform) and osteogenic zones in the scaffold holder with meticulous care. One angiogenic zone sandwiched between two osteogenic zones is considered as the structural unit of the 3D layered construct.

2.3.12.1 Characterization of the layered constructs

The morphology and structural organization of the layered constructs were evaluated using SEM after sectioning the layered constructs using cryotome (Leica, Germany). The compressive strength of the layered constructs was evaluated using a mechanical tester (Instron 4505) with 10KN load cell. The crosshead speed was set at 0.4 mm/min, and the load was applied until the scaffold was crushed. Averages of 5 measurements were used to compare the compressive strength of the constructs.

2.3.13 In vivo evaluation of the layered constructs

The ectopic animal model used in the present study has been reported elsewhere. All animal studies were carried out in accordance with the Institutional Animal Ethical Committee (IAEC) and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) (India). Layered constructs were subcutaneously implanted on athymic mice (Nude-HSD-Fox N1) for a period of 4 weeks and the extent of vascularization and 3D tissue formation was evaluated using histology.
2.3.13.1 Housing of nude mice

Experimental animals were brought from the central animal facility of Indian Institute of Science Bangalore on HEPA filtered cages in controlled atmosphere. Animals were transferred to Individually Ventilated Cage (IVC) systems (Citizen Industries, India) in the nude mice facility. Animals were housed in the IVC cage systems (Figure 2.3.2) in sterilized corncob bedding material. Sterile food pellets and water were provided *ad libitum* and feeding martial and bedding material were changed periodically under aseptic conditions.

Figure 2.3.2: Nude mice housing in the animal facility. A is the IVC cage racks with the cages. B and C are the pictures of nude mice in different magnifications.

2.3.13.2 Experimental groups

Constructs were categorized to 3 major groups viz., Test, where both osteogenic and angiogenic layers were bioengineered with cells, Control-A where only osteogenic layer is bioengineered with cells and Control-B where neither layers were bioengineered. Each group was sub-divided into two based on the angiogenic platforms. The flow chart of experimental groups is shown in Figure (2.3.3)
2.3.3 Subcutaneous implantation of the engineered constructs

Engineered layered constructs were prepared in the scaffold holders. Required number of constructs with scaffold holders was placed in 12-well plates containing endothelial growth media. Mice were anesthetised using the intramuscular injection of a combination of ketamine (35 mg/kg) + Xylazine (5 mg/kg) + Acepromazine (0.75 mg/kg). A subcutaneous pouch was surgically made on the left flank of the animal and the constructs were implanted aseptically in the surgical hood in the nude mice facility. Post surgical care has been given to all the implanted animals and was housed in the IVC system for the desired period of time.

2.3.13.4 Evaluation of the viability of osteogenic zones in vivo

The viability of cells in the osteogenic zones in vivo was evaluated 48 hrs after implantation. Animals were anesthetised and the constructs were taken out surgically and placed in pre-warmed NM in a falcon tube. Live dead assay was performed using the live/dead assay kit as previously described in section (2.2.10).

2.3.13.5 Evaluation of vascularization and 3D tissue formation in vivo

28 days after the implantation of the constructs, animals were sacrificed using overdose of anesthetics and the constructs were surgically removed and stored in 10% neutral buffered formalin solution. Constructs were processed and sections from the middle of the constructs using precision cutter (Buehler, Isomet 5000). Sections containing the centre points were stained with haematoxylin-eosin dyes.
and were imaged. Vascularization and tissue in-growth was analysed and quantified using image analysis software Image J (National Institute of Health).

2.3.14. EDAX analysis of the nano-fibrous layer for mineralization.

At 4 weeks of *in vivo* implantation, the layered constructs were surgically separated and EDAX analysis was performed as previously described (method 2.1.7.3).

2.3.15 Statistical Analysis

All results were obtained from triplicate samples. Quantitative results are represented as mean± standard deviation. Student’s t-test was performed to determine statistical significance in results. p value of less than 0.05 (p < 0.05) was considered to be statistically significant.

**References**


