CHAPTER 9

Studies on the chitosan-collagen-hyaluronic acid based scaffolds for bone regeneration

9.1 Introduction

Tissue engineering has emerged as a promising alternative approach in the treatment of malfunctioning or lost organs. In this approach, a temporary scaffold is needed to serve as an adhesive substrate for the implanted cells and physical support to guide the formation of the new organ. Beside the cell adhesion, cell growth, migration and retention of differentiated cell function, scaffold should be biocompatible, biodegradable and highly porous with large surface/volume ratio. As scaffolds are considered as the *in vitro* replacement for the *in vivo* extracellular matrix (ECM), mimicking the ECM cues for the scaffold preparation and modification should improve their performance. This school of thought mimicking the *in vivo* scenario has led to the emergence of the biomimetic approach of tissue engineering.

Yannas *et al* (1982) and Dagalakis *et al* (1980) conducted pioneering studies on collagen-glycosaminoglycan scaffolds to induce the regeneration of dermis of skin, sciatic nerve and knee meniscus. Chemical cross-linking by glutaraldehyde has been proposed to control the stability and degradation rate of these matrices, whereas porosity has been changed by both chemical and physical techniques. (Olde Damink *et al*., 1995; Barbani *et al*., 1995).
ECM that is defined as the acellular material surrounding the cell mainly consists of proteins and polysaccharides or glycosaminoglycans. Since ECM plays an important role in cell adhesion, proliferation, migration and differentiation (Ode et al., 2010), its physical and chemical properties are adapted for designing the biomimetic scaffolds. Von der Mark et al proposed three different strategies for achieving this goal: (1) Biomaterials releasing bioactive molecules. (2) Biomaterials coated with adhesive ECM proteins and peptide. (3) Biomaterials with nanoscale pattern inducing specific cell responses.

Identifying the ideal cell source, the in vivo microenvironment and understanding the regulatory mechanisms of their growth and differentiation play a significant role in determining the successful performance of a tissue engineered construct. Mesenchymal stem cells with its excellent properties like self-renewal, high expansion potential and osteogenic differentiation potential (Pal et al., 2009; Tae et al., 2006) is considered as the most prospective candidate for cell based tissue engineering for bone regeneration. Hyaluronic acid (HA) is one of the major glycosaminoglycans found in the ECM of fibroblasts. ECM include heparin (HEP), heparan sulphate, chondroitin sulphate, dermatan sulphate (DS) and keratan sulphate.

Hyaluronic acid (HA) is one of the major glycosaminoglycan (GAG) found in the ECM of mesenchymal stem cells (Ashhurst et al., 1990). They are involved in cell migration and differentiation and appear first in the ECM during tissue repair (Yamane et al., 2005). HA also plays a fundamental role during embryonic development and in wound healing, both in adult and fetal life stages, favouring cell migration processes (Soranzo et al., 2004). Our earlier study on the effect of major GAGs on proliferation and osteoblast differentiation human bone marrow derived MSCs (hMSCs) showed enhanced mineralization on HA treated tissue culture plates (Chapter 6). HA treated plates showed more secreted bone matrix with the highest amount of calcium.
The ECM of bone is composed of organic and inorganic matrix. Calcium phosphate in the form of hydroxyapatite forms the major part of the inorganic matrix whereas collagen type 1 is the major component of the organic matrix of bone. Type I collagen of the bone matrix serves as the template for the structural integration of the hydroxyapatite crystals, and along with the mineral matrix, contribute to the structural and mechanical properties of the bone. Collagen that promotes cell proliferation is widely used in tissue engineering, especially bone tissue engineering applications (Keogh et al., 2010).

Chitosan is a natural biomaterial, which is non-toxic, biocompatible and biodegradable. It has structural similarity to hyaluronic acid of ECM. It is also known to accelerate wound healing, influence tissue regeneration and osteogenesis (Yang et al., 2009; Venkatesan and Kim, 2010). Chitosan has been widely tested for bone and tissue engineering (Rochet et al., 2009; Chesnutt et al., 2009).

Based on our previous data demonstrating the beneficial effect of the ECM protein, collagen type 1 (Chapter 5), ECM- glycosaminoglycan (GAG), hyaluronic acid (Chapter 6) and a natural polymer, chitosan (Chapter 7) in supporting hMSC culture and enhancing their differentiation to osteogenic lineage, we have successfully developed a two-dimensional triopolymer coating demonstrating their synergistic effect on osteoblast differentiation and mineralization (Chapter 8). Our data showed that when chitosan, collagen type 1 and hyaluronic acid were used in a 1:1:1 ratio combination there was significant enhancement in mineralization with high amount of calcium with respect to the individual component treated and untreated plates.

In this study we have used chitosan; collagen type 1 and hyaluronic acid develop a biomimetic three dimensional culture system for hMSC culture and osteoblast
differentiation of hMSCs. Our aim was to optimize the concentration and the method of preparation of this tripolymer combination scaffolds to evaluate its structural stability and osteogenic potential in comparison to a simple 3% chitosan scaffold.

9.2 Materials and methods

9.2.1 Preparation of chitosan scaffolds

Chitosan solution (3% w/v) was prepared by autoclaving chitosan powder (>87.61% degree of deacetylation, which was a kind gift from Indian Sea Foods (Cochin, India) in distilled water and then dissolving it by adding 0.3M sterile glacial acetic acid (Merck, India). The chitosan solution at different concentrations (1%, 2% and 3% chitosan) was poured into the wells of a six well plate and frozen at −80 ºC overnight. The samples were lyophilized in a Freeze Dryer (Alpha 2D Plus, Martin Christ, Osterode, Germany) until completely dry. The sides of the samples were removed and the core was cut into 5mm diameter cylinders using a sharp punch. The samples were rehydrated in a graded ethanol series. For rehydration, the samples were equilibrated in two changes each of 100%, 95%, 80% and 70% ethanol solution for 60min respectively. The samples were incubated in the second 70% ethanol solution for 24h at 4 ºC. The samples were treated as sterile hereafter. The rehydration was continued with 60% and 50% ethanol solutions. After the final change of 50% ethanol, the samples were transferred to sterile DPBS. The samples were then incubated in antibiotic-antimycotic solution at 4 ºC for 48h. Prior to the cell culture, the scaffolds were incubated overnight in phenol red containing cell culture media in a humidified incubator at 37 ºC and 5% CO₂ in the air.
9.2.2 Preparation of tripolymer scaffolds

Tripolymer scaffolds were prepared using 1% solution of chitosan, collagen type I and hyaluronic acid. 1% chitosan (CHI) solution was prepared in 0.1N sterile acetic acid as described above (Section 9.2.1). 1% collagen type I (COL) (Sigma Aldrich, St. Louis, MO, USA) solution was prepared by dissolving collagen powder in 0.1N sterile acetic acid. 1% hyaluronic acid (HA) solution was prepared by dissolving potassium salt of hyaluronic acid (Sigma Aldrich, St. Louis, MO, USA) in sterile distilled water.

To obtain the tripolymer mix, first chitosan and collagen solutions were mixed thoroughly on a vortex and then hyaluronic acid solution was added into it and vortexes again to get a uniform solution. Different tripolymer combinations were made by mixing different proportions of each solution (Table 9.1).

<table>
<thead>
<tr>
<th>Scaffold Label</th>
<th>CHI 10mg/ml</th>
<th>COL 10mg/ml</th>
<th>HA 10mg/ml</th>
<th>Ratio CHI:COL:HA</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1:1:1</td>
</tr>
<tr>
<td>S2</td>
<td>1</td>
<td>0.1</td>
<td>1</td>
<td>1:0.1:1</td>
</tr>
<tr>
<td>S3</td>
<td>1</td>
<td>1</td>
<td>0.1</td>
<td>1:1:0.1</td>
</tr>
<tr>
<td>S4</td>
<td>0.1</td>
<td>0.1</td>
<td>1</td>
<td>0.1:0.1:1</td>
</tr>
<tr>
<td>S5</td>
<td>1</td>
<td>0.1</td>
<td>0.1</td>
<td>1:0.1:0.1</td>
</tr>
<tr>
<td>S6</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1:1:0</td>
</tr>
<tr>
<td>S7</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1:0:1</td>
</tr>
</tbody>
</table>

Table 9.1 Composition of the tripolymer scaffolds.

The samples were poured into the wells of a 6-well plate and frozen at -80 °C overnight. The samples were then lyophilized until completely dry. The samples that formed relatively strong porous scaffolds were selected for further processing and cell
culture. The sides of the samples were removed and the core was cut into 5mm diameter cylinders using a sharp punch. The scaffolds were cross-linked by immersing the samples in 40% ethanol containing 50mM 2-morpholinoethanesulphonic acid (MES, Sigma Aldrich, St. Louis, MO, USA) (pH 5.5) and 33mM 1-ethyl-3-(3-dimethyl aminopropyl) carbodimide (EDC, Sigma Aldrich, St. Louis, MO, USA). After cross linking, the samples were washed in 0.1M Na₂HPO₄ (pH 9.1), 1.0M NaCl and distilled water respectively. The samples were then lyophilized until dry. The samples were sterilized by incubating them in 70% ethanol for 24h at 4 °C. These sterile samples were transferred to sterile DPBS. The samples were incubated in antibiotic- antifungal solution for at least 48h at 4 °C. Prior to the cell culture, the scaffolds were incubated in phenol red containing cell culture medium overnight in a humidified incubator at 37 °C and 5% CO₂ in the air.

9.2.3 Human mesenchymal stem cells (hMSCs) culture on the Scaffolds

Human MSCs were isolated from bone marrow of adult human donors after taking informed written consent by method mentioned under Section 4.3.5. The cells were grown in hMSC media (Table 4.2).

9.2.3.1 Preparation of PKH26 labelled cells

PKH26 red fluorescent cell linker kit for general cell membrane labelling was obtained from Sigma Aldrich (St. Louis, MO, USA). The kit contained 1mM ethanolic solution of PKH26 dye for staining and Diluent C for diluting the dye and preparing the cell suspension. A final concentration of 2µM PKH26 dye for 1 × 10⁷/ml cells in staining volume of 2ml was used for the staining procedure. Just before staining fresh 2X working solution of the dye was prepared by adding 4µl of the dye to 1ml of Diluent C in a polypropylene centrifuge tube and mixed well to disperse. Human MSC were trypsinized and the pellet was obtained by centrifugation at 1800rpm for 5min. The cells were re-
suspended in serum free basal media (KO-DMEM) and counted by trypan blue exclusion method. $2 \times 10^7$ viable cells were taken in a 15ml centrifuge tube and washed once with serum-free basal media. The cells were centrifuged at 400×g for 5min to obtain a loose pellet. The supernatant was aspirated carefully leaving no more than 25µl media. The 2X cell suspension was prepared by adding 1ml of Diluent C to the cell pellet. The cells were re-suspended with gentle pipetting, ensuring complete dispersion. Precautions were taken not to vortex the cells and not to let the cells stand in Diluent C for long periods. 1ml of 2X cell Suspension was rapidly added to 1ml of 2X dye solution and immediately mixed by pipetting. Final concentrations after mixing the indicated volumes will be $1 \times 10^7$ cells/ml and $2 \times 10^{-6}$M PKH26. The cell/dye suspension was incubated for 1–5min with periodic mixing. The staining reaction was stopped by adding an equal volume (2 ml) of serum (FBS) and further incubated for 1min to allow binding of the excess dye. The sample was diluted with an equal volume of complete medium (MSC media). The stained cells were then centrifuged at 400 × g for 10min at RT. The supernatant was carefully removed and the pellet was transferred to a fresh 15ml centrifuge tube. The cells were washed three times with 10ml each of complete culture medium (MSC media) at 400 × g for 5min each. After the final wash the pellet was re-suspended in 10ml of culture media for cell counting and viability assessment.

The scaffolds were seeded with PKH-26 labelled hMSC at a seeding density of 100000 cells per cylindrical scaffolds of 5mm of diameter. The scaffolds were placed in MPC treated non-adherent (Nalge Nunc International, Rochester, NY, USA) culture plates and maintained in MSC media. The plates were incubated in a humidified incubator at 37 °C and 5% CO₂ in the air. The plates were observed under a fluorescent microscope for the PKH-26 stained cells in the scaffolds.
9.2.3.2 Calcein-Propidium Iodide (PI) live-dead Assay

The scaffolds seeded with unlabelled cells were stained with cell-permeable calcein acetoxymethyl (calcein AM) and PI (Sigma Aldrich, St. Louis, MO, USA), after 24h for live-dead assay. For the staining, the culture medium was aspirated and fresh media containing 2μm calcein and 0.01mg/ml PI was added to the plate and incubated at 37 ºC for 10min. The stain was removed and the plates were washed three times with DPBS. Cells labelled with PKH26 were stained with calcein alone for tracking the viability of the cells seeded into the scaffolds. The plates were then observed under a fluorescent microscope.

9.2.3.3 Osteoblast differentiation studies on scaffolds

After 7 days of culture, the cells were directed to osteogenic lineage by maintaining the cell containing scaffolds in osteogenic induction media. The cells were tracked at different time points by fluorescent microscopy for PKH-26 staining. The viability of the cells was evaluated by calcein-PI or calcein-PKH26 fluorescent staining on day 14, 21 and 28 days of osteoblast differentiation. The day 14 scaffolds with the cells were placed in tissue culture treated chamber slides and the migrated cells were stained for fibronectin, osteocalcin and vimentin using anti human antibody raised in mouse (BD Pharmingen, San Jose, USA). The nucleus was stained with DAPI. The fluorescent stained cells were observed under a fluorescent microscope (Nikon Eclipse 80i) and analysed by the Q Capture Pro 6 software.

The scaffolds were washed three times with DPBS and treated with 0.25% trypsin for cell retrieval. The scaffolds were also treated with TRI reagent (Sigma-Aldrich) for RNA isolation. The scaffolds were placed in adherent tissue culture treated plates (BD Biosciences, USA) for studying cell migration from the scaffolds at different time points.
9.2.4 Characterization of the scaffolds

9.2.4.1 Scanning electron microscopy (SEM)

The lyophilized samples were mounted on a stub using adhesive carbon conductive tape and sputter coated with gold in a JEOL-JFC-1100E ion sputtering device. The samples were then observed in FEI Quanta 200 environmental scanning electron microscope (ESEM; with EDAX-EDS system.) and the pictures were captured.

9.2.4.2 Swelling measurements

Three dry scaffolds were weighed (W_d) and placed into phosphate-buffered saline (PBS) at RT for 10h. After removing the unabsorbed solution, the wet weight (W_w) of the scaffold was determined. The swelling ratio of the scaffold was defined as the ratio of the weight increase (W_w - W_d) to the initial weight (W_d) according to following equation:

\[
\text{Swelling ratio (\%) = } \left( \frac{W_w - W_d}{W_d} \right) \times 100
\]

9.2.4.3 SEM analysis for cell morphology

The scaffolds seeded with cells were processed for SEM for analysing the cell morphology and distribution. The samples were fixed in the fixation buffer containing 2.5% Gluteraldehyde (GA) and 4% Para Formaldehyde (PFA) in PBS for 4hr in RT or overnight at 4°C. The fixed samples were washed thoroughly with PBS dehydrated with serial ethanol washes as follows. The samples were placed in 30% ethanol for 10 min, 50% ethanol for 10 min, 70% ethanol for 10 min or overnight, 85% ethanol for 20 min, 95% ethanol for 20 min, 100% ethanol for 20 min, 100% ethanol for 20 min or overnight, 100% Acetone for 20 min and final wash of 100% acetone for 20min. The samples were immediately freeze dried. The samples were mounted on a stub using adhesive carbon conductive tape and sputter coated with gold in a JEOL-JFC-1100E ion sputtering device.
The samples were then observed in FEI Quanta 200 environmental scanning electron microscope (ESEM) with EDAX-EDS system. The pictures were captured at different magnifications to analyse the cell morphology.

### 9.2.4.4 Scanning electron microscopy for the cell morphology and element analysis

The scaffolds with cells were processed for SEM (Section 9.2.3) on day 14 of osteogenic induction for analysing the cell morphology, distribution and mineralization. The samples were then observed in FEI Quanta 200 environmental scanning electron microscope (ESEM) with EDAX-EDS system and pictures were captured. Element analysis for calcium (Ca) and phosphorous (P) were also done.

### 9.2.4.5 Haematoxylin and Eosin (H&E) staining of the scaffolds sections

Cross sections of the scaffolds were prepared and stained with H & E for visualising the cells deep within the scaffolds.

#### 9.2.4.5.1 Preparation of scaffold embedded wax blocks

The scaffolds containing the cells were fixed using buffer containing 2.5% gluteraldehyde (GA) and 4% Para formaldehyde (PFA) in PBS for 4hr at RT or overnight at 4°C. After, the fixation, the scaffolds were washed thrice with PBS and then dehydrated in a graded ethanol series (30%, 50% 70%, 95%, 100% and 100% ethanol) by keeping the scaffold in each solution for 5min at RT. After the ethanol treatment, the scaffolds were placed in two changes of xylene at 65 ºC for 5min each. Prior to embedding the scaffolds in the paraffin wax, the scaffolds were placed in a series of wax-xylene gradient (1:3, 1:1 and 3:1 wax: xylene at 65 ºC) for 30min each. Finally, the scaffolds were placed in 100% paraffin wax (at 65 ºC) for 30min. The molds for block preparation were set on ice and molten paraffin wax (65 ºC) was poured into the molds.
The scaffolds were then transferred to the wax filled molds and the blocks were allowed to solidify. Once solidified, the scaffold-embedded blocks were placed in a microtome and 5µm thick sections were made. The mid sections were fixed on a glass slides coated with egg white, dried at 37 °C and stained with Haematoxylin and Eosin (H & E).

9.2.4.5.2 H& E staining of the embedded scaffold sections

First, the slides were pre-warmed at 65 °C for 10min and were placed in three changes of xylene for 5min each at RT. Then the slides were rehydrated in a graded ethanol series (100%, 95%, 70%, 50%, and 30% ethanol) in Coplin jars, by placing or dipping the slides in the solutions for 1min each. The slides were then washed once with tap water (1min) and placed in haematoxylin stain for 1-5min. The slides were rinsed with tap water and placed in ammonia water for 1min. The slides were then placed in 70% ethanol for 1min and dipped in Eosin stain for 45sec-1min. The slides were rinsed once in 75% ethanol (1min), twice in 100% ethanol (1min each) and again twice in xylene (1min each). The slides were mounted with DPX. The stained sections were observed under a phase contrast microscope and pictures were captured.

9.2.4.6 ALP assay

Cell lysate was collected on day 14 of differentiation from various scaffolds and ALP assay was performed to evaluate the ALP activity (Section 5.2.3.2).

9.2.4.7 Immunofluorescent staining of the migrated cells for fibronectin

The day 14 old scaffolds with the cells were placed in tissue culture treated chamber slides and the cells migrated cells were stained for fibronectin using anti human antibody raised in mouse (BD Pharmingen, San Jose, USA). The nucleus was stained
with DAPI. The fluorescent stained cells were observed under a fluorescent microscope (Nikon Eclipse 80i) and analysed by the QCapture Pro 6 software.

9.3 Results

9.3.1 Chitosan Scaffolds

Chitosan formed porous scaffolds on lyophilisation. Among the different chitosan scaffolds, 3% chitosan formed the most strong and stable scaffold followed by 2% and 1% chitosan. All the chitosan scaffolds supported hMSCs culture in the form of aggregates and showed very few dead cells (Fig. 9.1).

**Figure 9.1 Human MSCs culture on chitosan scaffolds.** Calcein-PI staining demonstrating the cell distribution and viability of hMSCs on different chitosan scaffolds. The Figure insets (A, D, G) show the cell distribution on the scaffolds at a lower magnification. Human MSCs were seen as cell aggregates on chitosan scaffolds (A, D, G). PI staining showed very few (B, E, H) dead cells (white arrows). The number of cell aggregates was more on 3% chitosan scaffolds (C: Inset) followed by 2% (B: Inset) and 1% (A: Inset) chitosan scaffolds. Scale bar - 20µm.
The number of cell aggregates was more on 3% chitosan (Fig. 9.1C: Inset) followed by 2% (Fig. 9.1B: Inset) and 1% (Fig. 9.1A: Inset) chitosan scaffolds. Due to their structural stability and superior cell culture properties, 3% chitosan was selected for further evaluation.

9.3.2 Human MSC culture on chitosan scaffold

9.3.2.1 PKH26 labelling of hMSCs

Human MSCs were successfully labelled with PKH26. The cell membrane picked up the stain and imparted red fluorescence to the cells under a fluorescent microscope (Fig. 9.2). The staining protocol gave 100% efficient staining of the hMSCs.

![Figure 9.2 PKH26 labelled hMSCs. PKH26 labelled cells had red cell membrane (B, C). The phase contrast pictures are false stained (blue) for obtaining the composite image. Scale bar- 20µm.](image)

9.3.2.2 Tracking of the labelled hMSCs in the scaffolds

PKH26 labelled cells seeded into the scaffolds were tracked by fluorescent microscopy. Human MSCs penetrated into the scaffold pores and formed aggregates (Fig. 9.3). 48h post cell seeding showed large cell aggregates (Fig. 9.3D). Strong background fluorescence coming from the scaffolds indicated deep cell penetration. Calcein staining performed after 5 days of culture confirmed cell viability and proliferation of hMSCs on
3% chitosan scaffolds (Fig. 9.4). Calcein staining also demonstrated deep penetration of the cell aggregates in the porous scaffold.

![Fig. 9.3 Tracking of PKH26 labelled hMSCs on 3% chitosan scaffold.](image)

1h post-seeding showed large number of round single cells mainly on the surface of the scaffold (A). 48h post-seeding showed large cell aggregates deep within the scaffold (C). Presence of the cell aggregates deep within the porous scaffolds was evident from the difference in the focal length for different cell aggregates and blurred and background fluorescence (D). The cells on the scaffolds were either spherical single cells or cell aggregates. Scale bar - 20µm.

![Fig. 9.4 Human MSCs viability on 3% chitosan scaffold.](image)

Calcein staining showed viable PKH labelled hMSCs on 3% chitosan (CHI) scaffold after 5days of culture (D). Calcein staining also revealed either spherical single cells or cell aggregates on the scaffold (D) whereas the tissue culture plates (TCP) showed adherent spindle shaped cells (A). PKH26 and calcein staining showed large cell aggregates deep within the scaffold (D-F). Presence of the cell aggregates deep within the porous scaffolds was evident from the difference in the focal length for different cell aggregates and blurred and background fluorescence from the calcein-stained scaffold (D). Scale bar - 20µm.
9.3.2.3 Osteogenic potential of hMSCs on 3% chitosan scaffold

Live cultures of PKH26 labelled hMSCs, undergoing osteogenic differentiation on 3% chitosan scaffolds, were tracked at different time points by fluorescent microscopy (Fig. 9.5). Human MSCs showed formation of significantly large cell aggregates on 3% chitosan scaffolds maintained in osteogenic induction media. Scaffolds maintained in hMSC media showed relatively smaller cell aggregates. There was increase in the background fluorescence from day 14 to day 21 of culture that indicated cell proliferation and migration.

![Figure 9.5 Osteogenic differentiation of hMSCs on 3% chitosan scaffold.](image)

Calcein staining performed on day 21 of osteogenic induction showed viable PKH26 labelled hMSCs on 3% chitosan scaffolds (Fig. 9.6). Calcein staining also revealed formation of significantly large number of big cell aggregates in osteogenic induction media when compared to MSC media.
Figure 9.6 Viability of hMSCs undergoing osteoblast differentiation on 3% chitosan scaffold. PKH26 labelled hMSCs showing calcein positive viable cell aggregates on day 21 of osteogenic differentiation. The upper panel set (A-C) showed cells grown in hMSC (hMSC) media and the lower panel set (D-F) showed the cells in osteogenic induction media (OST). More number of large cell aggregates was observed on Scaffold in OST media (D-F). Scale bar - 20μm.

Immunofluorescent staining of the cells migrated from 14days old 3% chitosan scaffolds maintained in hMSC media showed the expression of vimentin and fibronectin (Fig. 9.7). The cells did not show the expression of osteocalcin (Fig. 9.10I-L). Immunofluorescent staining of the cells that migrated from the 14days old 3% chitosan scaffolds in osteogenic media (OST), showed the expression of vimentin, fibronectin and osteocalcin (Fig. 9.7). Difference in the staining intensity and morphology of cells were observed with the vimentin positive cells of hMSC and OST media. All the cells migrated from the scaffold of MSC media were positive for vimentin and showed a spindle shaped fibroblastic morphology. Not all the cells migrated from the scaffold of OST media were positive for vimentin and the vimentin positive cells rather showed a flattened morphology (Fig. 9.7D). High expression of fibronectin was present around the cells migrated from OST media (Fig. 9.7H). There was higher intensity of staining and well-defined clear structures whereas, the cells migrated from MSC had lesser fibronectin expression and rather showed a very hazy structure ((Fig. 9.10H).
Figure 9.7 Immunofluorescent staining of the cells from the 3% chitosan scaffolds. The cells migrated from the 14 days old scaffold in hMSC media (A-L) shows expression of vimentin (VM; A-D) and fibronectin (FN; E-H). The cells did not show any expression of osteocalcin (OCN; I-L). The cells migrated from the 14 days old scaffold in osteogenic media (A-L) show expression of vimentin (VM; M-O), fibronectin (FN; P-S) and osteocalcin (OCN; T-W). The composite micrographs are shown at higher magnification (D, H, L, O, S, W). Note the higher expression of FN in osteogenic media (S). The nucleus is stained with DAPI (blue). Scale bar – 20µm.
9.3.3 Tripolymer scaffolds

Tripolymer combinations were formulated based on our two dimensional plate-coating studies. S1 and S3, which formed good, strong and porous scaffolds, were selected for further characterization and osteogenic potential evaluation (Fig. 9.8). S1 had equal proportion of CHI, COL and HA (1:1:1; S1) whereas S3 had less amount of HA (1:1:0.1; S3). The tripolymer scaffolds were compared to 3% CHI scaffolds for their structural and functional characteristics. The CHI (1%) and HA (1%) formed clear transparent solutions whereas COL formed a translucent solution (Fig. 9.8A). The S1 combination formed a very heterogeneous solution due to the precipitation caused by the addition of HA and the heterogeneity was less in S3 combination solution (Fig. 9.8 B, C). The solutions formed porous scaffold on lyophilisation and were cut into 5mm diameter cylindrical scaffolds for further studies (Fig. 9.8E-G).

Figure 9.8 Preparation of tripolymer scaffolds. 1% polymer solutions (A) and the polyblend solutions (B-D) used for the scaffold preparation. S1 (B) and S3 (C) were heterogeneous solutions. Macroscopic view of the final porous scaffolds (E-G) used in the experiments.
9.3.4 Characterization of the scaffolds

9.3.4.1 Microstructure

The SEM images showed various pore shapes and sizes for each of the scaffolds (Fig. 9.9). Chitosan scaffold showed highly porous structure having macro and micro pores and there was homogenous distribution of pores. The tripolymer scaffold, S1 showed heterogeneous structure and pore distribution, whereas, in the S3 scaffold, where the amount of hyaluronic acid was less, the microstructure changed significantly. Unlike S1 scaffold, S3 scaffold had moreover homogenous microstructure.

![Figure 9.9 Scanning electron micrographs depicting the porous structure of different scaffolds. S1 scaffold had very heterogeneous pore structure and distribution (A, D) whereas S3 had more or less homogenous structure. Three percentage chitosan scaffolds showed a homogenous microstructure (C) with macropores (red outline) and micropores (green outline) (F). The macropores were 100-200µm in diameter and the micropores were 20 -30µm in diameter. Scale bar -500µm (100x), 200µm (200x).](image)

9.3.4.2 Swelling ratio

The stability of the scaffold was evaluated by determining the swelling ratio after soaking in physiological fluid (Fig. 9.10). All the samples could bind 500 to 900-fold of
physiological fluid and still maintain their form stability. The cross-linked tripolymer scaffolds (S1, S3) showed less swelling than the non-cross linked 3% chitosan scaffolds. The swelling ratio decreased as the proportion of hyaluronic acid increased in the tripolymer composition scaffolds.

**Figure 9.10 Swelling fold.** Swelling fold (%) of the scaffolds soaked in physiological fluid. The values indicate mean ± SD. 3% chitosan scaffold showed significantly higher swelling fold than S1 and S3 scaffolds. S3 scaffold had relatively higher swelling fold than S1 scaffold. (n=3).

### 9.3.5 Human MSC culture on tripolymer scaffolds

The hMSCs were labelled with PKH26 before seeding the scaffolds and were tracked at different time points of culture. The fluorescent micrographs taken after 24h of hMSCs seeding into the scaffolds showed spindle shaped adherent fibroblast like cells in S1 and S3 tripolymer scaffolds (Fig. 9.11). Three percentage chitosan scaffolds did not show any adherent fibroblast population, but rather showed spherical cells and large cell aggregates. There was cell penetration deep into the pores of the scaffolds. Trypsinization
did not give good cell yield from any of the scaffolds, as there was poor cell dissociation from the scaffolds.

Figure 9.11 PKH26 labelled hMSC tracking in the scaffolds after 24h of seeding. HMSCs on scaffold S1 (B) and S3 (E) scaffolds shows adherent population having spindle-shaped fibroblast like morphology. Human MSCs on 3% chitosan (H) shows spherical cells and large cell aggregates. The cells penetrated deep into all the scaffolds which were evident from the background fluorescence and different focal planes (C, F, I). Scale bar - 20µm.

The calcein-PI staining performed after 24h of seeding showed more than 90% live cells on all the scaffolds (Fig. 9.12). Calcein also revealed spindle shaped fibroblast like cells on S1 and S3 scaffolds; whereas large spherical cell aggregates were visible in 3% chitosan scaffolds. The strong background fluorescence coming from the scaffolds indicated the presence of cells deep within the scaffolds.
Figure 9.12 Calcein-PI viability assays after 24h of seeding. Unlabelled hMSCs seeded into the scaffolds were stained with calcein and PI after 24h of incubation. hMSCs on scaffold S1 (A) and S3 (D) scaffolds showed large number of live (green) adherent populations having spindle shaped fibroblast like morphology. Human MSCs on 3% chitosan (CHI, G) showed large cell aggregates having live cells. PI (red) showed very few numbers of dead cells in all the scaffolds (B, E, H). The cells penetrated deep into all the scaffolds which were evident from the high background fluorescence coming from the scaffolds (C, F, I). Scale bar - 20µm.

9.3.6 Evaluation of the osteogenic potential of the scaffolds

The PKH26 labelled cells were observed under a fluorescent microscope at different stages of osteoblast differentiation (Fig. 9.13). Cells were visible throughout the differentiation process. Adherent fibroblastic cells were observed on S1 and S3 scaffolds. Both the scaffolds showed increase in cell proliferation and migration from day 7 to day 14 of osteogenic induction. This was evident from the increase in the total fluorescence as well as the background fluorescence. S3 scaffold maintained in osteogenic media showed patches of cells with high intensity fluorescence on day 14 of osteogenic induction (Fig. 9.13K).
Human MSCs on S1 (A, D, G, J) and S3 (B, E, H, K) scaffolds showed similar adherent fibroblastic morphology, whereas 3% chitosan (3% CHI, C, F, I, L) showed cell aggregates. Note the increase in fluorescence from day7 to day 14 and the more fluorescence from the scaffolds maintained in osteogenic media (OST) than the hMSC media (hMSC). Day 14 showed patches of cells (K) with high intensity fluorescence on S3 scaffold maintained in OST media. Scale bar – 20µm.

9.3.7 Cell viability

The viability of the cells at the end differentiation (day 14) was evaluated by calcein staining which stained live cells (Fig. 9.14). DAPI staining revealed the nucleus of the cells and showed the cell distribution.
Large number of adherent spindle shaped cells and high background fluorescence from the scaffolds were visible on S1 and S3 scaffolds. The nuclear stain DAPI also demonstrated large number of cells in the scaffolds. There was no significant difference in cell number or distribution observed with cells in hMSC media and osteogenic (OST) media (Fig. 9.12). 3% chitosan showed many cell aggregates of viable cells (Fig. 9.14). Scaffold from osteogenic media had larger aggregates than the scaffold from hMSC media.

The morphology of the hMSCs on different scaffolds after 14 days of osteoblast differentiation was assessed by calcein staining evaluation at higher magnification (Fig. 9.15). S1 and S3 showed adherent spindle shaped fibroblast like cells in both hMSC and OST media; whereas, hMSCs were seen as aggregates on 3% chitosan scaffold in both...
hMSC and OST media. Human MSCs grew as cell aggregates on 3% chitosan scaffold (Fig 9. 15). There were many spots with hazy fluorescence on the scaffolds indicating the fluorescence coming from deep within the scaffold. There was increase in the fluorescence from day 7 to day 14 of osteogenic induction.

![Figure 9.15 Human MSC morphology on different scaffolds: Calcein staining](image)

Figure 9.15 Human MSC morphology on different scaffolds: Calcein staining. Calcein staining performed on day 14 of osteogenic induction revealed spindle shaped fibroblast like morphology on S1 (A, D) and S3 (B, E) scaffolds in both hMSC (A, B) and osteogenic (OST) (D, E) media. Human MSCs formed cell aggregates on 3% chitosan scaffolds in both hMSC (C) and OST media. Scale bar - 20µm.

9.3.8 SEM analysis for cell morphology on the scaffolds

The SEM images taken after 24h of cell seeding for the evaluation of cell morphology confirmed the morphological difference in the cells seeded into the triopolymer (Fig. 9.16) and 3% chitosan (Fig. 9.17) scaffolds. S1 scaffold showed patches of adherent spindle shaped cells in layers inside the porous scaffold (Fig. 9.16A). Higher magnification (5000x) showed cells with smooth surfaces (Fig. 9.16B). S3 scaffold also showed adherent spindle shaped cells (Fig. 9.16C). There were thick layers of cells that appeared in patches. Higher magnification (5000x) revealed smooth surface of the cells (Fig. 9.16D).
Figure 9.16 Scanning electron micrographs depicting the morphology of hMSCs on S1 and S3 scaffolds. The cells showed spindle shaped fibroblastic morphology and were seen in layers on both the scaffolds (A, C; scale bar -100µm). The higher magnification of the white squire area marked in (A, C) is shown as (B, D) respectively. The cells on both the scaffolds had a smooth surface (B, D; scale bar – 10µm).

Human MSCs showed a spherical morphology on 3% chitosan scaffolds (Fig. 9.17). They showed an irregular surface and had filopodia (Fig. 9.17C). Cell aggregates of 3-4 cells were also seen on SEM analysis (Fig. 9.17D). Though the PKH26 (Fig. 9.11) cell tracking and calcein staining (Fig. 9.12) showed large number of large cell aggregates on 3% chitosan scaffolds, only few cells were found on SEM.
Figure 9.17 Scanning electron micrographs depicting the morphology of hMSCs on 3% chitosan scaffold. The cells had spherical morphology (A, scale bar -100µm). (B) depicts the higher magnification (10µm) of the white square area marked in (A) and (C) depicts the higher magnification (Scale bar -5µm) of the white square area marked in (B). The cells had an irregular surface (C). Cell aggregates were also seen on the 3% scaffolds (D).

9.3.9 Evaluation of osteoblast differentiation and mineralization by SEM analysis

SEM analysis was done on day 14 of osteoblast differentiation for the evaluation of cell morphology and presence of mineral deposits (Fig. 9.18-9.20). S1 scaffold showed adherent fibroblast like cells that covered the entire surface of the scaffold in both hMSC and OST media (Fig. 9.18). Mineral like deposits were observed on the cells in OST
media, which had rather an irregular surface. Human MSCs maintained in hMSC media had a smooth surface.

Figure 9.18 SEM analysis of hMSCs on S1 scaffold on day 14 of osteogenic induction. The right side panel figures show the white squire marked area of the left side figures in higher magnification. SEM revealed spindle shaped fibroblast like morphology for hMSCs on S1 scaffold. The cells covered the entire surface of the scaffold (A, C). Human MSCs had a smooth surface in hMSC media. Cells in osteogenic (OST) media showed mineral like deposits and the cells had an irregular surface. Scale bar- 100µm (A, C), 10 µm (B, D).

S3 scaffold also showed adherent fibroblast like cells that spread over the entire surface of the scaffold in both hMSC and OST media (Fig. 9.19). Cells showed a smooth surface in both the media, but small mineral-like deposits were visible in OST media.
Figure 9.19 SEM analysis of hMSCs on S3 scaffold on day 14 of osteogenic induction. The right side panel figures show the white square marked area of the left side figures in higher magnification. SEM revealed spindle shaped fibroblast like morphology for hMSCs on S3 scaffold. Layers of cells were present covering the entire surface of the scaffold (A, C). Human MSCs had a smooth surface in both hMSC and the osteogenic (OST) media. Spots of mineralization (C, D black arrows) were visible in OST media. Scale bar- 100µm (A, C), 10 µm (B, D).

SEM analysis after 14 days of osteogenic induction showed hMSCs as cell aggregates in 3% chitosan scaffold (Fig. 9.20). Although PKH26-tracking (Fig 9.13) and calcein visualized many cell aggregates (Fig 9.14), only very few cells were seen by the SEM. Cell aggregates in both hMSC as well as OST media had irregular surface. Cell aggregates of OST media showed large amount of mineral like deposits on the surface (Fig. 9.20D).
Figure 9.20 SEM analysis of hMSCs on 3% chitosan scaffold on day 14 of osteogenic induction. The right side panel figures show the white square marked area of the left side figures in higher magnification. SEM showed hMSCs in aggregates on 3% chitosan scaffolds in hMSC as well as OST media (A, D). The cells had irregular surface (B, E). Cell aggregates of OST media showed mineral like deposits all over the surface. Scale bar- 100µm (A, C), 10 µm (B, D).

Element analysis for calcium (Ca) and phosphorous (P) by SEM showed elevated Ca and P levels on the scaffolds (Fig. 9.21). S1 scaffold showed in hMSC media showed the presence of Ca but there was no detectable amount of phosphorous. The cells in OST media had higher level of Ca (3.5%) than the cells in hMSC media (1.1%). In contrast, S1 scaffolds showed higher level of calcium (4.5%) in hMSC media than OST media (0.4%); but the amount of phosphorous was more in OST media (3.5%) than hMSC media (0.5%). 3% chitosan scaffolds showed higher amount of calcium (2.1%) and phosphorous (4.8%) in the scaffolds maintained in osteogenic media (OST).
Figure 9.21 Element analysis for calcium and phosphorous using EDAX-EDS system. S1 scaffold showed 1.1% calcium on cells in hMSC media (A). But a higher level was present on cells in osteogenic (OST) media (3.5%; D). S3 scaffold in OST media showed higher amount of P (3.2%) and lower amount of Ca (0.4%) than in hMSC media (P-0.5%, Ca-4.5%). 3% chitosan scaffolds showed detectable amount of Ca and P only in the scaffolds maintained in OST media (Ca-2.1%, P-4.8%). Since most of the cell aggregates were lost during SEM sample processing, the element analysis values may not be accurate on 3% chitosan scaffolds.
9.3.10 H & E staining of the scaffold sections

H & E stained the nucleus blue, the cytoplasm pink and the scaffold material red. Presence of cells deep within all the tested scaffolds (S1, S3, 3% chitosan) was revealed by the H & E staining (Fig. 9.22).

Figure 9.22 H & E staining of scaffold sections on day 14 of osteogenic induction. All the scaffolds showed the presence of cells with blue nucleus and pink cytoplasm (Yellow arrows) within the scaffold. The scaffold material was stained red. S3 scaffold demonstrated the presence of large number cells in both hMSC (C) and OST (D) media. 3% chitosan scaffold showed cells in compact groups (E, F). Scale bar - 20µm.

Large number of cells with blue nucleus and pink cytoplasm were seen in the scaffold pores of S1 (Fig. 9.22A, B) and S3 (Fig. 9.22C, D). 3% chitosan showed comparatively
less spread out cells and most of the cells appeared in groups suggestive of cell aggregates (Fig. 9.22E, F).

9.3.11 ALP assay

ALP assay done on day 14 of osteoblast differentiation showed increase in the ALP activity for both the cells in osteogenic and non-osteogenic medium when compared to the baseline ALP level of the starting undifferentiated hMSC population (Fig. 9.23). There was significant increase (2-8 fold) in ALP expression by the cells in the osteogenic media compared to the cells in the non-osteogenic (hMSC) media. Cells of 3% chitosan scaffold in OST media showed 2-fold higher ALP activity than S1 and S3 scaffolds.

![Figure 9.23 ALP assay](image)

**Figure 9.23 ALP assay.** ALP assay was carried out on day 14 of osteogenic induction. The dotted line represents the level of ALP in the initial undifferentiated population. Note the significant increase in the ALP activity of the cells in scaffolds maintained in osteogenic (OST) media. Cells undergoing osteogenic differentiation in 3% chitosan scaffold showed significantly higher ALP level than S1 (**p<0.01) and S3 (*p<0.05) scaffolds.
9.3.12 Immunofluorescent staining for fibronectin

Day 14 scaffolds, when placed on tissue culture treated adherent chamber slides showed PKH26 labelled cells migrated from the scaffolds to the slides (Fig. 9.24).

**Figure 9.24 Immunofluorescent staining of the migrated cells for fibronectin.** The PKH26 labeled cells migrated from the scaffolds and grew as adherent populations on tissue culture plates (red fluorescence). There was more expression of fibronectin (green fluorescence) by the cells migrated from scaffolds maintained in osteogenic media (OST; F, N, V) than the cells in hMSC media (A, J, R) on all the scaffolds. DAPI stained the nucleus blue. The composite figure of PKH26, fibronectin and DAPI is also shown here (D, H, L, P, T, X). Scale bar - 20µm.
All the cells migrated from the scaffolds grew as adherent fibroblast like cells. When these cells were stained for fibronectin, the cells migrated from the scaffolds maintained in the osteogenic media (OST) showed higher expression when compared to the cells migrated from scaffolds maintained in hMSC media. These cells from OST media showed well-defined fibronectin structure with high intensity staining. The cells from hMSC media showed a faint staining depicting very hazy fibronectin structure. There was no significant difference in the fibronectin expression among the different scaffolds. Figure 9.23 also showed the nucleus stained blue with DAPI and a composite figure depicting the expression of PKH26, fibronectin and DAPI.

9.4. Discussion

In this study, we have prepared chitosan based scaffolds and evaluated its potential for hMSC, culture and osteogenic differentiation. We have standardised PKH26 staining for cell tracking and calcein –PI staining for cell morphology and viability in scaffolds. The aim of this study was to formulate a novel composite scaffold consisting of various natural polymers like chitosan, collagen type1 and hyaluronic acid for the application in bone tissue engineering and compare its properties and osteogenic potential with a simple chitosan based scaffold.

Chitosan is widely used in tissue engineering applications due to its excellent properties such as biocompatibility, biodegradability and it can be moulded in to various forms. Although chitosan has no toxic effects on cells, the cell culture properties of chitosan depends on many factors like the source of chitosan (Nwe et al., 2009), degree of deacetylation (Freier et al., 2005), the molecular weight, the concentration of chitosan (Mathews et al., 2011b) and above all the type of cells. Chitosan has shown to support the adhesion of many cells like mouse fibroblasts (Nwe et al., 2009), neurons
(Freier et al., 2005), rat bone marrow MSCs (Cho et al., 2008) and osteoblastic cells (Hsieh et al., 2005). Though chitosan based gels and scaffolds are widely used for hMSCs based tissue engineering, not many studies are there reporting the effect of pure chitosan on hMSCs adhesion, proliferation and differentiation. Our previous studies (Chapter 7) show that the coating density of chitosan influences the adhesion of hMSCs to chitosan treated culture plates. At higher coating density (>100µg/ cm²) chitosan did not support cell adhesion but stimulated the formation of cell aggregates.

As we used higher concentration of chitosan (1-3%) for the scaffold preparation, hMSCs did not adhere to the scaffold surface. Like in the two dimensional (2D) culture, hMSCs formed cell aggregates on the chitosan scaffolds. Interestingly the number and size of the cell aggregates increased as the concentration of chitosan increased. This may be due the more structural stability and stronger cell attachment inhibition associated with higher concentration of chitosan. From the calcein-PI cell viability assay, PKH26 cell tracking, we identified 3% chitosan scaffold ideal for hMSC culture. In addition, we confirmed the stability of hMSC culture in both hMSC and osteogenic media (OST) for 21 days without losing their integrity and viability on 3% chitosan scaffold. The morphological difference observed with the migrated cells from OST media with respect to hMSC media also indicated the possible differentiation to osteogenic lineage. In addition, the expression of osteocalcin and higher fibronectin by the cells migrated from the scaffold in OST media also shows the osteogenic potential of 3% chitosan scaffolds.

Poor mechanical strength and cell compliance of chitosan has led to the modification of chitosan with other compounds to improve its strength or cell adhesive property or both (Di Martino et al., 2005; Huang et al., 2005). The biomimetic modification of chitosan by incorporation COL (showing excellent hMSC adhesion) and HA (having high osteogenic potential) have showed significant enhancement of the
osteogenic differentiation of hMSCs (Chapter 8). In that study, we identified the
tripolymer combination of 1:1:1 mixing ratio of CHI, COL and HA possessed the best
osteogenic potential. Again in this study, we have modified the CHI scaffold by blending
COL-I and HA at different concentrations to evaluate the change in hMSC adhesion and
osteogenic differentiation. Out of five tripolymer combinations, we selected two scaffolds
S1 and S3 for further analysis based on the structural stability and strength.

The pore size, distribution, porosity and shape influence the cell adhesion, growth,
phenotype and properties (Ma et al., 2003). SEM analysis showed significant difference
in the pore shape, size and distribution among S1, S3 and 3% CHI scaffolds. Doillon et al
(1986) reported that the technique used and the viscosity of the material solution affects
the mean pore size of the scaffold. The EDC cross-linking and re-lyophilisation also
affect the microstructure of the scaffolds. Since 3% CHI was a homogenous solution, it
formed highly porous scaffolds with homogenous pore distribution. However, as the
S1and S3 tripolymer solution was heterogeneous, the microstructure of the S1 scaffolds
had irregular pore shapes and heterogeneous distribution of pores. The S3 scaffold had
more over a sheet like microstructure. Ma et al (2003) had reported that repeated freezing
could promote the collagen fibres to combine again, thereby causing consolidation of
some small pores, into sheets. The proportion of hyaluronic acid in the tripolymer
scaffolds significantly changed the microstructure of the scaffolds as observed with S1
and S3, which varied only for hyaluronic acid.

The water retaining property of the scaffold can greatly influence their efficacy in
tissue engineering applications. The ability of the scaffold to retain water is dependent on
many factors like the hydrophilicity of the material, porosity of the scaffold and chemical
cross-linking (Shanmugasundaram et al., 2001). The behaviour of the scaffold during
swelling influences the cell adhesion, distribution and nutrient-waste exchange. CHI,
COL and HA have good water binding capacity which was reflected in their triopolymer combination also. Thus, the scaffolds projected a favourable condition for cell adhesion and proliferation within the scaffold. 3% non-cross-linked chitosan scaffold showed high swelling ratio of 900%. During the tripolymer scaffold fabrication, the EDC cross-linking increased the hydrophobicity and the re-lyophilisation, which decreased the porosity, resulted in lower swelling ratio for S1 and S3 scaffolds. The increase in the degree of cross-linking reduced the swelling ratio of the scaffolds (Shanmugasundaram et al., 2001). Equal amount of chitosan, collagen type 1 and hyaluronic acid in S1 scaffold resulted in higher degree of cross-linking than S3, which had unequal proportions of the polymers. This explains the lower swelling ratio of S1 over S3 scaffolds.

All the tested scaffolds provided favourable environment for hMSC culture. They showed good cell viability and retention inside the scaffold. SEM analysis for cell morphology revealed spherical shaped cells and cell aggregates having coarse surface on chitosan scaffold. Amaral et al (2006) observed that MG-63 osteoblast-like cells from human osteosarcoma attached and spread with long filopodia and numerous cell-to-cell contacts on chitosan scaffolds with lower degree of acetylation (DA, 13%) but cells tended to remain spherical and to grow into spheroid-like cellular aggregates on chitosan scaffolds with higher DA (30%). Based on their findings, they concluded that, the DA played a key role in determining the affinity of cells towards the 3D substrates, possibly by controlling the nature of the initial adsorbed protein layer.

Most of the cells and cell aggregates, which were unbound or loosely bound to the 3% chitosan scaffold material, were washed away during SEM sample processing. The cells, which remained in the scaffold, had filopodia, which is used to attach to the substratum. The biomimetic modification of chitosan scaffolds with COL and HA dramatically changed the cell culture properties of the scaffold. The triopolymer scaffolds
permitted the adhesion and proliferation of hMSCs. The cell morphology and growth characteristics of hMSCs on CHI scaffolds resembled the in vivo scenario. There were more cell-cell interactions and the cell aggregates provided a 3D growth. Unlike 3% chitosan scaffold, the tripolymer scaffold showed adherent spindle shaped cells, the characteristic feature of hMSCs in an in vitro culture system. Collagen is reported to promote hMSC adhesion and proliferation (Liu et al. 2004), HA also supports hMSC adhesion and together they modified the scaffold surface property by altering the adsorbed protein layer which mediates cell adhesion. The chitosan component in the tripolymer scaffold increased the strength and stability of the scaffold.

The experiments to evaluate the osteogenic potential of the scaffolds gave promising results. Human MSCs were able to proliferate on all the tested scaffolds. PKH26 tracking showed cells on the scaffolds, and with time, they increased in number and penetrated the scaffolds going deeper and deeper. Calcein staining confirmed the good viability of the cells even after 14 days of culture in the scaffolds. Increase in ALP activity and mineral like deposits by the cells in osteogenic media confirmed the osteogenic potential of the scaffolds. The ECM secreted by osteoblasts contains fibronectin, which is required for further differentiation and survival of the osteoblasts (Globus et al., 1998). Therefore, the presence of fibronectin surrounding the cells indicates the presence of differentiating or differentiated osteoblasts. Our experiments to demonstrate migration of differentiated cells from the scaffolds showed prominent expression of fibronectin around the cells migrated from scaffolds maintained in osteogenic medium for 14 days. This indicates the presence of osteoblast in the scaffold and their ability to migrate to the surrounding environment. This will have implication in bone tissue engineering where scaffolds containing differentiated cells are applied to the site of injury or fracture.
The inorganic mineral matrix of bone is made up of calcium and phosphate in the form of hydroxyapatite. The SEM analysis revealed mineral matrix like deposits on all the scaffolds in OST media with 3% chitosan showing cell aggregates with prominent deposits all over the surface. Mineral deposits were more obvious on S1 scaffold than S3, which on element analysis showed 3% higher calcium. Mineral matrix on 3% chitosan scaffold also showed detectable amount of both calcium and phosphorous.

The increase in the ALP activity and remarkable mineral deposits by the cells in osteogenic media on 3% scaffolds projected its superior osteogenic potential. This may be due to the three dimensional cell growth and differentiation of the cell aggregates that mimicked the in vivo tissue growth. Since most of the cells and cell aggregates were lost during SEM sample preparation, the exact picture of cell and cell aggregate distribution and mineralization were poorly understood. As chitosan is cheaply available and can easily be made into porous scaffolds by simple standard protocol, pure chitosan scaffold could be further explored for bone tissue engineering applications.

Our tripolymer scaffolds, which had three components with known osteogenic property, also favoured hMSC culture and osteoblast differentiation. Since the scaffolds had large surface area, they were able to support the adhesion and growth of large number of cells. The 24h post seeding SEM images showed only patches of cells on the scaffolds and after 14 days of culture the SEM image showed whole scaffold surface covered with layers of cells. PKH26 cell tracking, calcein staining, H & E staining and SEM analysis proved the penetration, proliferation, differentiation and survival of cells deep within the scaffold. This indicates the biocompatibility and cell conductivity of the scaffold and their possible application in tissue engineering.
We have demonstrated the ability of the undifferentiated and differentiated cells to migrate from the scaffold and grow in favourable environment. This is one of the most desirable characteristic features for in vivo applications like cell delivery to the site of injury or healing bone fractures. Though there was an expected high cell yield from the scaffolds owing to the large surface area and enhanced cell proliferation, the trypsinization process gave very poor cell yield. This was the main disadvantage of all the tested scaffolds in the in vitro culture system. S1 and S3 scaffolds had large number of cells in multi layers, which thus had strong cell-cell and cell-material interactions, providing not a favourable environment for the action of trypsin. Although hMSCs were not attached or loosely attached to chitosan scaffolds, trypsinization was unsuccessful in dissociating the cells from the cell aggregates. The strong cell-cell and cell-ECM interaction must have shielded the cell aggregates from the action of trypsin.

To summarise, we have developed stable biomimetic CHI-COL-HA based scaffolds, S1 (1CHI: 1 COL: 1 HA) and S3 (1CHI: 1 COL: 0.1 HA) which supported and promoted hMSC adhesion and osteoblast differentiation. Cells grew and differentiated as adherent fibroblastic population on S1 and S3 scaffolds whereas the cell grew as aggregates on 3% chitosan scaffold. 3% chitosan scaffold showed more swelling ratio, homogenous porosity and higher ALP activity and mineral like deposits compared to S1 and S3. Our results also suggested that, when the culture conditions mimicked the in vivo scenario and the cells were grown as aggregates rather than adherent fibroblast like population, there was enhancement of osteoblast differentiation. All the three tested scaffolds showed excellent cell compatibility and good osteogenic potential and thus hold great promise in hMSCs based bone tissue engineering.