Chapter 4: RESULTS AND DISCUSSION

For the ease of understanding and for better clarity this chapter is divided into four sections. Each of the first three sections will describe the results of experiments performed on single nanofiller containing composite nanofibrous scaffold. The last section in this part of the thesis will give a comparative analysis among the composites from the bone tissue engineering perspective.

4.1 Embedded silica nanoparticles in PCL nanofibrous scaffolds enhanced osteogenic potential for bone tissue engineering

4.1.1 Research question

To what extent will the incorporation of nSiO2 in the PCL nanofibrous scaffold increase the mechanical strength and osteogenic potential compared to PCL?

4.1.2 Hypothesis

Incorporation of nSiO2 in PCL nanofibrous scaffold will yield a scaffold which is significantly stronger mechanically and having higher osteogenic potential compared to PCL.

4.1.3 Morphological and Chemical Characterizations of PCL/nSiO2 nanocomposite fibrous scaffolds

4.1.3.1 Scaffold Architecture

SEM images of PCL (Fig. 4.1.1A, 4.1D), PCL/4%nSiO2 (Fig. 4.1.1B, 4.1E) and PCL/8%nSiO2 (Fig. 4.1.1C, 4.1.1F) nanocomposite scaffolds are shown. The obtained nanofibers were randomly oriented and were in the range of 150-350nm in diameter.
Figure 4.1.1  **PCL/nSiO₂ composite scaffolds with nanofibrous structure:** Scanning electron micrographs showing (A, D) PCL, (B, E) PCL/4% nSiO₂ & (C, F) PCL/8% nSiO₂ scaffolds with fibers of diameter around 150-350nm.

TEM images of PCL/nSiO₂ show that in some cases the nanoparticles are partially embedded into the fiber surface (Fig. 4.1.2A). In other cases, the nanoparticles are fully embedded and aggregated within the fibers (Fig. 4.1.2B) and are apparently not distributed within all fibers.

Figure 4.1.2 **TEM of PCL/nSiO₂ electrospun nanocomposite scaffolds:** TEM images showing A) PCL/4%nSiO₂ and B) PCL/8%nSiO₂ nanofibrous scaffolds

The incorporation of nSiO₂ was confirmed using FTIR (see Fig. 4.1.3), where the peak at 1100cm⁻¹ is attributed to Si–O–Si stretching vibration, and another peak at 950cm⁻¹ indicated
Si–terminal non-bridging vibration.\textsuperscript{109, 110} These peaks were absent in the native PCL scaffold.

Figure 4.1.3 FTIR of mineralized PCL/nSiO\textsubscript{2} composite scaffolds: FTIR spectra showing (A) bare PCL/4\% nSiO\textsubscript{2} (B) bare PCL/8\% nSiO\textsubscript{2} (C & D) mineralized PCL/4\% nSiO\textsubscript{2} and PCL/8\% nSiO\textsubscript{2} scaffolds after 21 days in SBF and (E) bare PCL scaffold.

4.1.3.2 Contact Angle Measurement

The contact angle was measured in order to check the hydrophilicity/ hydrophobicity of the scaffold. PCL on its own had a contact angle of 146.2\° ± 1.12 (Fig. 4.1.4A), whereas the fabricated composite scaffolds showed a lesser contact angle, namely, 118.22\° ± 0.29 and 114.01\° ± 0.133 respectively for PCL/4\% nSiO\textsubscript{2} and PCL/8\% nSiO\textsubscript{2} scaffolds after 21 days in SBF (Fig. 4.1.4B & 4.1.4C). The measured contact angle value reflects the hydrophilicity of the electrospun scaffold with respect to milli-Q water. The decrease in contact angle clearly indicates the fall in the hydrophobicity of PCL in the composite. This decreased hydrophobicity may aid in better protein adsorption and hence in the cell attachment.
Figure 4.1.4  **Contact angle measurements of electrospun composite scaffolds**: Images showing contact angle with water of A) PCL, B) PCL/4% nSiO$_2$ and C) PCL/8% nSiO$_2$ scaffolds.

4.1.3.3 Mechanical testing

Stress at failure of electrospun native PCL scaffold was found to be $0.43 \pm 0.02$ MPa, and that of PCL/4% nSiO$_2$ was $0.47 \pm 0.02$ MPa (Fig. 4.1.5A). Stress at failure of PCL/8% nSiO$_2$ scaffold was as high as $0.95 \pm 0.01$ MPa, which is more than twice ($p<0.05$) that of both PCL and PCL/4%nSiO$_2$ fibrous scaffolds. Strain to failure values of PCL, PCL/4%nSiO$_2$ and PCL/8%nSiO$_2$ are $33.43 \pm 0.24$, $36.42 \pm 1.9$, and $26.08 \pm 0.5$ respectively (Fig. 4.1.5B).

Using 10MPa as the strength of fully dense PCL$^{111}$ and 100 MPa as the strength of fully dense hydroxyapatite (HA)$^{112}$, the percentage increase in strength by HA additions based on a rule of mixtures approach is 10% for 4% w/w addition of silica and 20% for 8% w/w silica addition. In our results we observed approximately 10% increase in strength with 0.5 w/v silica which is consistent with our calculations, however, the increase in strength due to 1.0 w/v addition of silica was as high as 120%. Addition of 10 nm size silica particles to 150-350 nm PCL fibers appears to behave in a continuum sense for very low volume fractions, but at the level of 2.2 vol. % the strength increase was much more substantial than based on continuum calculations. Later results will show that there was also a very substantial increase in biological properties at the 8% nSiO$_2$ (2.2 vol.% silica) additions (see below).
4.1.3.4 In vitro biomineralization studies

Figure 4.1.6 shows the SEM images of in vitro biomineralization studies of the prepared composite scaffolds in SBF solution. The SEM images showed that the amount of mineralization was higher in PCL/nSiO\textsubscript{2} scaffold when compared to pristine PCL. The mineralization was found to be better in scaffold with 8\% nSiO\textsubscript{2} (Fig.4.1.6G & H) than 4\% nSiO\textsubscript{2} (Fig. 4.1.6D & E).

The EDS spectra confirmed the deposition of calcium phosphate on the composite scaffolds (Fig. 4.1.6C, F & I). The Ca/P ratio of PCL/4\% nSiO\textsubscript{2} and PCL/8\% nSiO\textsubscript{2} scaffolds were found to be 1.71 ± 0.085 and 1.67 ± 0.064, respectively, suggesting that HAp may have been formed. The Ca/P ratio of mineral deposits on PCL fibers without the nSiO\textsubscript{2} was 1.33 ± 0.014 indicating that mineral deposits formed in the absence of nSiO\textsubscript{2} may not be nHAp.

The mineralization on PCL/4\%nSiO\textsubscript{2} and PCL/8\%nSiO\textsubscript{2} was characterized by FTIR also. The non-mineralized PCL/nSiO\textsubscript{2} scaffolds (Fig. 4.1.3A & B) show no peaks corresponding to phosphate. The phosphate peaks were found at 564 cm\textsuperscript{-1} in PCL/4\%nSiO\textsubscript{2} scaffold (Fig. 4.1.3C) and 571 cm\textsuperscript{-1} in PCL/8\%nSiO\textsubscript{2} scaffold (Fig. 4.1.3D). The band at 603 and 571 cm\textsuperscript{-1} corresponds to γ\textsubscript{4} of phosphate mode\textsuperscript{113} The peak at 1034 cm\textsuperscript{-1} was assigned to ν\textsubscript{3} PO\textsubscript{4} vibrations and the peak at 1432 cm\textsuperscript{-1} was due to calcium phosphate\textsuperscript{114, 115} These results confirm mineralization in the composite scaffolds.
Figure 4.1.6 SEM images and EDS graphs of mineralized PCL/nSiO₂ composite scaffolds: SEM images of mineralized scaffolds after 21 days in SBF, (A & B) PCL scaffold, (D & E) PCL/4% nSiO₂ scaffold and (G & H) PCL/8% nSiO₂ scaffolds. EDS graphs of mineralized scaffolds after 21 days in SBF (C) PCL (F) PCL/4% nSiO₂ scaffolds and (I) PCL/8% nSiO₂ scaffolds showing the Ca/P ratios.

From the mineralization results in Fig. 4.1.6 (comparing Fig. 4.1.6H to Fig. 4.1.6B and 4.1.6E) it was evident that there was a qualitatively greater increase in the extent of mineralization at the 2.2 vol.% silica addition compared to native PCL or the PCL with 1 vol.% silica addition. This may be explained on the basis of the greater anticipated surface coverage of the silica nanoparticles on PCL fibers when the content of silica is increased. The silica particles could act as nucleation sites for HA p formation as has been suggested previously. The Ca/P ratio of the mineral deposits were the same in the silica containing scaffolds and also were found to be similar to that of the HA p in physiological condition in bone. However, in the PCL native scaffold the mineral deposits were not hydroxyapatite (observed Ca/P ratio was 1.33). Thus silica influences both the chemistry and the extent of mineralization on the scaffolds.
4.1.4 Isolation and characterization of hMSCs from umbilical cord blood (UCB)

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

![FACS analysis of isolated hMSCs](image)

**Figure 4.1.7** FACS analysis of isolated hMSCs: The FACS analysis data shows positive expression for surface markers (A) CD29 (B) CD44 (C) CD73 while negative expression for (D) CD31 (E) CD34 and (F) CD45. Flow cytometric dot plots in the top panel showing, 3.3, 77.1 and 94.6% positive for CD29, CD44 and CD73 respectively.

4.1.5 Cell viability and proliferation studies

The cytocompatibility of the composite scaffolds were checked by direct contact method. The cell viability studies were also performed with nSiO_2 (0.5% and 1%) alone. AlamarBlue
assay results show that nSiO$_2$ by itself (that is, without the scaffold) was toxic to cells with only 30% viability (Fig. 4.1.8B) compared to its control (cells alone) while composite scaffolds with the same concentration of nSiO$_2$ showed more than 85% cell viability compared to the control (PCL scaffold) after 48h (Fig. 4.1.8A). The proliferation studies on the PCL/4%nSiO$_2$ (Fig. 4.1.8C) indicated that the proliferation of stem cells was not changed by the presence or absence of nSiO$_2$. Further the cell proliferation regime was observed to be upto day 7. After day 7 the cells were in a quiescent state with no increase in cell number.

Figure 4.1.8  Cell Viability of PCL/nSiO$_2$ scaffolds and nSiO$_2$ powder: Cell viability of hMSCs on (A) PCL/4% nSiO$_2$ and PCL/8% nSiO$_2$ scaffolds and (B) nSiO$_2$ powder compared to their respective controls after 48h. (C) Proliferation on PCL/nSiO$_2$ scaffolds up to 21days. Value represents mean ± S.D of three independent experiments (n = 3).
4.1.6 Silica uptake by hMSCs using flow cytometry

After 6h incubation of hMSCs with the nSiO\textsubscript{2} particles and PCL/nSiO\textsubscript{2} scaffolds, cells were analyzed by flow cytometry. The side scattering of all the samples was compared. The cells that were in contact with bare nSiO\textsubscript{2} particles (Fig. 4.1.9A & Fig. 4.1.9B) showed more granularity and side scattering compared to cells alone (Fig. 4.1.9C), whereas cells in both 4% and 8% concentrations of nSiO\textsubscript{2} particles in PCL did not show much side scattering (Fig. 4.1.9 D, 4.1.9 E). Side scattering was also absent when cells were tested on bare PCL scaffolds without nSiO\textsubscript{2} (Fig. 4.1.9F). This is indirect evidence that the nSiO\textsubscript{2} particles when added alone are taken up into the cell interior, whereas when present as part of the scaffold the silica nanoparticles did not enter the cells.

![Figure 4.1.9 FACS analysis of cells treated with bare nSiO\textsubscript{2} and nSiO\textsubscript{2} containing scaffold.](image)

Uptake of particles was clearly shown by the FACS analysis as well as ICP-AES analysis\textsuperscript{119,120} (Fig. 4.1.9 and Table 4.1.1). However, the same concentration of nSiO\textsubscript{2} when suitably embedded in the polymer scaffold, showed no cellular uptake (Fig. 4.1.9 and Table 4.1.1). The embedding of the nanoparticles into the nanofibers enabled exploitation of the positive aspects of silica while protecting the cells from their potential toxicity. The absence of
toxicity of the nSiO$_2$ containing scaffold argues against a surface related mechanism of toxicity, for then both bare nSiO$_2$ as well as the scaffolds containing nSiO$_2$ should have shown toxicity.

4.1.7 Silica uptake by hMSCs using ICP-AES

ICP-AES analysis shows that only the hMSCs that were in contact with bare nSiO$_2$ particles showed silicon content. The cell lysates from cells incubated with 0.5% and 1% nSiO$_2$ showed 0.65 ppm and 3.04 ppm of silicon respectively (Table 4.1.1), while the cell lysate from cells which were in contact with PCL, PCL/4%nSiO$_2$ and PCL/8%nSiO$_2$ scaffolds show < 0.1 ppm silicon content which is below the detectable limit of ICP-AES. (Table 4.1.1).

<table>
<thead>
<tr>
<th>Samples</th>
<th>Amount of silicon in cell lysate (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cells</td>
<td>BDL*</td>
</tr>
<tr>
<td>cells + 4% nSiO$_2$</td>
<td>0.65</td>
</tr>
<tr>
<td>cells + 8% nSiO$_2$</td>
<td>3.04</td>
</tr>
<tr>
<td>Cells + PCL</td>
<td>BDL</td>
</tr>
<tr>
<td>cells + PCL/4% nSiO$_2$</td>
<td>BDL</td>
</tr>
<tr>
<td>cells + PCL/8% nSiO$_2$</td>
<td>BDL</td>
</tr>
</tbody>
</table>

* BDL: Below detectable limit (0.01)

This result supports the side scattering studies and is additional evidence that bare nSiO$_2$ is taken up into the cells, but such uptake is absent when the nSiO$_2$ is associated with the PCL nanofibers. With reference to the TEM pictures of Fig. 4.1.2, this is because the nSiO$_2$ is either partially or fully embedded into the fibers and are not free to be taken up by the cells when in the scaffold.

Despite the benefits of silica as a biological material in terms of mineralization and protein adsorption it is evident from the present results that they are also cytotoxic. In the free state,
bare nanosilica powder showed 70% cell death when exposed to hMSCs (Fig. 4.1.8). The mechanism of toxicity can be attributed to the ingestion of the nanoparticles by the cells\textsuperscript{121}.

4.1.8 Cell morphology on scaffolds

Cell adhesion studies showed that the hMSCs were attached to the scaffold in 6h and were well spread after 12h in culture (Fig. 4.1.10). In both the PCL/nSiO\textsubscript{2} nano-composite scaffolds the morphology of the cells were flattened, sheet like, elongated with filopodia like extensions as seen in SEM image.

![SEM images of hMSCs attached on A & B) PCL, C & D) PCL/4\% nSiO\textsubscript{2}, E & F) PCL/8\% nSiO\textsubscript{2} nanofibrous scaffolds after 12h of incubation showing well spread morphology.](image1)

Fluorescent staining with DAPI showed that hMSCs were evenly distributed and attached throughout the scaffold (Fig. 4.1.11a, 4.1.11b). These results did not appear to be affected by the presence or absence of nSiO\textsubscript{2} in the fibers.
Figure 4.1.11 Fluorescence microscope images showing nuclei of hMSCs stained with DAPI: Images showing (A) PCL, (B) PCL/4% nSiO₂ and (C) PCL/8% nSiO₂ scaffolds, at 10x magnification after 24h of incubation in culture.

4.1.9 Protein adsorption studies

The protein adsorption of the composite scaffolds incubated in culture media at different time intervals were assessed by BCA assay and the results are shown in Fig. 4.1.12. This clearly indicates that the incorporation of nSiO₂ into the scaffolds resulted in an increase in the protein adsorbed. The maximum adsorbed protein levels were found to be on the PCL/8%nSiO₂ scaffold.
Figure 4.1.12 Protein adsorption on PCL and PCL/nSiO$_2$ composite scaffolds: Graph showing total protein adsorbed on PCL, PCL/4% nSiO$_2$ and PCL/8% nSiO$_2$ scaffolds after 2-6h duration. Value represents mean ± S.D of three independent experiments (n=3). (*shows p< 0.05)

The extent of protein adsorption was increased by 48% to 158% (when compared to pure PCL) when the nSiO$_2$ containing scaffolds were soaked in FBS from 2-6 hrs (see Fig. 4.1.12). Substantial adsorption of protein on silica nanoparticle surfaces has been shown earlier indicating that both curvature and particle size and surface area play a role in protein adsorption on silica nanoparticles. In the current system, introduction of the silica nanoparticles did not alter the fiber size or shape (see Fig. 4.1.12), however, any silica exposed on the surface of the fibers would represent a region of high surface area and a region with a chemistry favouring protein binding. The difference in protein adsorption between the two silica loading levels may be explained in terms of the amount of silica exposed on the fiber surfaces, although this may not entirely explain the steep increase in adsorption when going from 4% w/w to 8% w/w silica in PCL.

4.1.10 Differentiation of hMSCs on PCL/nSiO$_2$ scaffold

Differentiation was measured by ALP activity (normalized with the total protein) as plotted in figure 4.13 for the various scaffolds (PCL alone and PCL with 4% and 8% nSiO$_2$) in both
normal and osteogenic media. The cells showed a basal level of ALP activity in all scaffolds in the proliferating phase of hMSCs (that is, less than 7 days). After 7 days, during the quiescent phase, there was a marked increase in ALP activity, particularly for the case of the PCL-8% nSiO₂ scaffolds.

Figure 4.1.13 The alkaline phosphatase activity in hMSCs on composite scaffolds: ALP activity of hMSCs on PCL, PCL/4% nSiO₂ and PCL/8% nSiO₂ scaffolds in normal medium and osteogenic medium over time (1-21 days). (n=3)

The osteogenic differentiation of hMSCs on the scaffold was further confirmed by the qualitative analysis of RUNX2 protein in the hMSCs. RUNX2 is a protein which is localized to the nuclei of osteoblasts. Immunocytochemistry showed the presence of RUNX2 localized within the nuclei of the hMSCs on PCL/nSiO₂ scaffolds, at Day 7, 14 and 21 (Fig.4.1.14) in osteogenic medium. Consistent with the ALP activity results, there was observed expression of RUNX2 after 7 days. Figure 4.1.14 shows the merged images of the scaffold (green), nuclei (blue) and RUNX2 (red) antibody staining. In the merged image, blue and red staining in the nuclei are seen as pink (refer supplementary information for the individual images).
Figure 4.1.14 Immunocytochemistry for RUNX2 on hMSCs on scaffolds: Fluorescent images showing hMSCs on (A, B & C) Day 1, (D, E & F) Day 7, (G, H & I) Day 14 and (J, K & L) Day 21 in osteogenic medium on scaffolds PCL, PCL/4%nSiO₂ and PCL/8%nSiO₂ scaffolds respectively.

In Figure 4.1.13, the differentiation of hMSCs into an osteoblastic lineage (measured by their ALP activity) was not observed in the proliferation regime of the cells (less than 7 days) but only in the quiescent regime (greater than 7 days - compare to Fig 4.8C). This finding correlates to earlier findings, that ALP activity is lower at the proliferation stage of hMSCs\textsuperscript{123, 124}. In the quiescent regime, nSiO₂ enhanced differentiation but more so in the osteogenic medium. The combination of osteogenic medium and the presence of nSiO₂ gave the maximum differentiation potential. This is evidence that the silica particles may themselves be differentiation promoters to an osteoblastic lineage. We also checked the expression of RUNX2 which is a known osteogenic marker. The immunocytochemistry results for the expression of RUNX2 in the cells on the PCL, PCL/4%nSiO₂ and PCL/8%nSiO₂
nanocomposite scaffolds at various time points (Fig. 4.1.14) give a molecular evidence that differentiation is primarily limited to the quiescent phase after day 7. This is the first study that suggests that hMSCs may differentiate more readily when exposed to embedded silica. More study may be required to better understand the mechanistic role of embedded silica on hMSC differentiation. Bare silica nanoparticles alone cannot be used as promoters because of their toxicity resulting from their ingestion by cells; what is critical is the need to contain the silica nanoparticles in a suitable embedding medium to realize their positive biological potential. This was successfully achieved by incorporating nSiO$_2$ nanoparticles in PCL nanofibers.

The bare nSiO$_2$ particles were found to be toxic to hMSCs. Eventually as the scaffold degrades, the nSiO$_2$ particles could be released from the scaffold, which may cause toxic effects in the long run. In order to get over this problem we thought of incorporating a silicate nanofiller “NC”, so that we could get a hold of the positive properties of silica in the form of silicate and yet avoid the toxicity involved.

### 4.2 In vitro evaluation of electrospun PCL/nanoclay composite scaffold for bone tissue engineering

#### 4.2.1 Research Question

Will the PCL/NC scaffold be biocompatible and to what extent can the NC increase the strength and osteogenic potential of PCL scaffold.

#### 4.2.2 Hypothesis

The incorporation of nanoclay into the PCL nanofibrous scaffolds form a biocompatible, mechanically strong scaffold with significantly high osteogenic potential compared to PCL scaffold.

#### 4.2.3 Intercalation of NC in complete medium

Intercalation of NC in complete medium was done by sonicating. The dried NC was tested by XRD analysis. The NC after intercalation procedure did not show any change in the XRD pattern compared to the non-intercalated NC (Fig.4.2.1)
From the product of the NC intercalation procedure in medium, electrospinning was performed along with PCL. Nanofibers were obtained by electrospinning but many beads were observed when analyzed by SEM (Fig. 4.2.2). This further showed that the intercalation of NC did not take place on using complete medium.

Figure 4.2.1  X-ray diffraction after intercalation of NC in medium: Graph showing XRD patterns of NC and PCL/NC nanocomposite after intercalation

Figure 4.2.2  SEM of fibers electrospun after intercalating NC in medium: Images showing a) lower magnification b) higher magnification of the beads along with fibers in the PCL/NC scaffold.
4.2.4 Intercalation of NC in polymer

PCL/NC film was prepared and the dispersion of NC in PCL polymer matrix was detected by XRD analysis. Figure 5.3 compares the XRD patterns of NC powder, PCL, and solvent-cast PCL/NC films prepared with different NC concentrations (1, 2, 4 & 6 wt %). Layered silicate in NC exhibits a characteristic peak at $2\theta \sim 4.88^\circ$ and PCL has a scattering peak at $2\theta \sim 21.4^\circ$ and $23.8^\circ$. However, the characteristic peak of silicate disappeared in the XRD pattern of all the prepared PCL/NC nanocomposites (Fig.3). This may be mainly because of large spacing between the layers making it an exfoliated nanocomposite or due to the absence of ordering after the preparation process$^{125}$. The sharp peaks of PCL at $20\sim21.4^\circ$ and $23.8^\circ$ were retained in all its nanocomposites but with reduced intensity as compared to bare PCL. The peak width, however, was not affected which indicated that the degree of crystallinity of PCL was not changed by the incorporation of NC at least in the thin films unlike in previous studies$^{126}$ in more bulk materials.

![X-ray diffraction patterns of PCL, PCL/NC composites](image)

**Figure 4.2.3** X-ray diffraction patterns of PCL, PCL/NC composites: Graph showing XRD patterns of PCL, NC and PCL/NC nanocomposites
4.2.5 Electrospun nanofibrous composite scaffolds

PCL/NC nanofibrous scaffolds were characterized using SEM. Figure 5.4 shows the SEM micrographs of electrospun PCL/NC with clay concentrations of 1%, 2%, 4% and 6%, having diameters in the range of 200-700 nm.

Figure 4.2.4 PCL/NC composite nanofibrous scaffolds: SEM micrographs showing nanofibrous structure of a) PCL b) PCL/1% NC c) PCL/2% NC d) PCL/4% NC e) PCL/6% NC scaffolds.
It is well known that nanofibrous scaffolds fabricated by electrospinning method attempt to mimic the extracellular matrix, hence offering a significant increase in surface area for cellular interaction and influence many aspects of cell behavior such as morphology and functionality\textsuperscript{127–130}. The incorporation of NC in PCL yielded, uniform, randomly oriented nanofibers (Fig. 4.2.4b, c, d & e). Such fibrous structure would result in a large surface area-to-volume ratio and interconnected porosity. The PCL/6%NC scaffold seemed to have higher fiber diameter compared to the other scaffolds (Fig. 4.2.4e & 4.2.5). These factors are considered essential for cellular growth in vitro and in vivo as they are directly involved in the transport of oxygen and nutrients to the cells.\textsuperscript{131}

### 4.2.6 Mechanical testing

The mechanical properties of the prepared nanocomposite scaffolds were evaluated by measuring their tensile stress and young’s modulii. Tensile stress measurements (Fig.4.2.6) suggested that addition of 4 and 6 % NC resulted in tensile stress value greater than PCL, with 6% NC showing maximum strength. Similarly, Young’s modulus values (Fig.4.2.7) showed that addition of NC leads to greater strength compared to native PCL, thereby enhancing the mechanical compatibility of the nanocomposites. Although there is a good improvement in the Young’s modulus of nanocomposites when compared to pure PCL, the
trend (Fig. 4.2.7) seems to be consistent with increase in NC concentrations showing almost similar values for all the four nanocomposites. These results illustrate that, even though there is a marked rise in the tensile stress values of the nanocomposites with increasing NC loadings, the increase in NC content does not have much effect on the Young’s modulii of the nanocomposites. Despite the fact that the scaffolds with higher concentration (6%) of NC were found to be better substrates to provide enhanced mechanical strength, these results did not correlate to the better biological properties which will be discussed later.

**Figure 4.2.6**  Tensile stress measurements: Graphs showing tensile stress values of PCL and PCL/NC nanocomposite scaffolds (p<0.05).

**Figure 4.2.7**  Young’s modulus measurement: Graphs showing the young’s modulii of PCL scaffold and PCL/NC nanocomposite scaffolds.
4.2.7 Contact angle measurement

The contact angle measurements of PCL and PCL/NC nanocomposite scaffolds with milli-Q water were performed. Pure PCL is hydrophobic in nature. Results of contact angle measured with water shows that NC decreased the hydrophobicity of the scaffolds to a certain extent (i.e. decrease in contact angle), with increase in NC weight percentage (Table 4.2.1). Therefore, surface wettability of the scaffold is enhanced by NC inclusion, which in turn can aid in cell attachment, spreading and well defined cytoskeletal organization.132

Table 4.2.1 Contact angle values of nanofibrous scaffolds: Data showing contact angle of PCL, PCL/4%NC and PCL/6%NC electrospun nanofibrous scaffolds with distilled water.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Contact Angle (θ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCL</td>
<td>146° ±1.5</td>
</tr>
<tr>
<td>PCL/4%NC</td>
<td>135.48° ± 1.6</td>
</tr>
<tr>
<td>PCL/6%NC</td>
<td>129.12 ± 1.2</td>
</tr>
</tbody>
</table>

4.2.8 Serum protein adsorption

The amount of total protein adsorbed onto the nanofibrous scaffolds were evaluated using BCA assay. Proteins adsorbed onto the surface of the substrate, either from serum or secreted by cells are believed to mediate cell attachment, migration, and growth. Protein adsorption test was conducted in order to evaluate the biological properties of the scaffolds.133 It was found that the nano-fibrous architecture along with NC addition enhanced the amount of protein adsorbed (Fig.4.2.8). As the time period of incubation increases, the concentration of protein adsorbed increased showing maximum adsorption at 6h, which then gradually started dropping at 8h (Fig.4.2.8) as reported earlier39. Higher protein adsorption was noticed in 4% NC containing scaffolds. Such increment in protein adsorption in 4%NC scaffold may be related to the difference in surface composition of the nanofibrous scaffolds134, 135. Other reasons include high surface area contributed by some of the exposed NC and also due to the fine surface morphology of the fibers. While in PCL/6%NC scaffolds had a higher fiber diameter and hence lower surface area leading to a reduced protein adsorption compared to PCL/4%NC scaffolds as shown in the following calculation. Hence PCL/6%NC showed 4/5th of the protein adsorbed on PCL/4%NC nanofibrous scaffold.

\[ \rho L\pi r^2 = W \ldots \ldots (1) \]
W = weight of scaffold
ρ = density of the material
L = length of the fibers
r = radius of the nanofibers
\[ A = 2\pi rL \] ......(2)

where, A = surface area

From equation (1) and (2),
\[ A = 2\pi r \times \left( \frac{W}{\rho \pi r^2} \right) \]
\[ \therefore A = \left( \frac{2W}{\rho} \right) \times \left( \frac{1}{r} \right) \]
\[ \therefore A \propto \left( \frac{1}{r} \right) \]

\[ \therefore \left[ \frac{\text{A of PCL/6\%NC}}{\text{A of PCL/4\%NC}} \right] = \left[ \frac{\text{r of PCL/4\%NC}}{\text{r of PCL/6\%NC}} \right] \]
\[ \therefore \left[ \frac{\text{A of PCL/6\%NC}}{\text{A of PCL/4\%NC}} \right] = \frac{200\text{nm}}{250\text{nm}} \]
\[ \therefore \left[ \frac{\text{A of PCL/6\%NC}}{\text{A of PCL/4\%NC}} \right] = \frac{4}{5} \]

Since, Protein adsorbed \( \propto \) A of scaffold,
\[ \therefore \text{Protein adsorbed on PCL/6\%NC} = \left( \frac{4}{5} \right) \times \text{(Protein adsorbed on PCL/4\%NC)} \]

**Figure 4.2.8** Protein adsorption on PCL and PCL/NC scaffolds: Graph showing total protein from serum adsorbed on PCL, PCL/4\%NC, PCL/6\%NC after 2h, 4h, 6h and 8h of incubation with serum containing medium(p < 0.05).
4.2.9 *In vitro* biomineralization studies

Bioactivity of a scaffold material is assessed in vitro by checking the ability to mineralize when immersed in SBF. Figure 4.2.9 shows the SEM micrograph of mineral deposits on the surface of PCL and PCL/NC scaffolds after 21 days of mineralization. The mineral deposition process was better in both the nanocomposite scaffolds (4% and 6% NC) when compared to pure PCL scaffold. After 21 days of immersion in SBF, all fibers were fully covered by apatite like layer (Fig. 4.2.9 b & c). Thus, the incorporation of NC promoted apatite formation on nanofibrous scaffold demonstrating the bone forming ability of the material. One of the major attribute to this is due to some of the NC tubes exposed on the fiber surface which serves as the nucleation site for apatite formation\textsuperscript{136}.

![SEM of mineralized scaffolds](image)

**Figure 4.2.9 SEM of mineralized scaffolds:** Images showing a) PCL b) PCL/4%NC c) PCL/6%NC mineralized scaffolds incubated in SBF after 21 days.

4.2.10 Viability of hMSCs on PCL/NC scaffolds

hMSCs isolated from cord blood were used in our study for cellular characterization of the scaffolds, because of their expandability, availability and their potential to differentiate into osteoblasts\textsuperscript{137}. alamar Blue assay was carried out to check the viability of hMSCs after 24h on PCL and PCL/NC nanofibrous scaffolds. Viability of hMSCs grown on various PCL/NC scaffolds were compared with hMSCs grown on control PCL scaffolds. As shown in figure10, PCL/NC scaffolds showed higher percentage viability with respect to PCL scaffolds after 24h, indicating that the NC containing scaffolds support cell survival better than the control PCL.
Figure 4.2.10 Viability of hMSCs on PCL and PCL/NC scaffolds: Data showing 24h cell viability on control PCL, PCL/2%NC, PCL/4%NC and PCL/6%NC significantly higher than PCL (p<0.05).

Despite the fact that there is a decreasing pattern in viability with increase in NC content, percentage of cell viability in composites was comparatively higher than on PCL scaffolds. This shows that addition of NC does not impart any toxicity to the cells grown on the substrate and hence are cytocompatible. Aforementioned results also revealed that among various PCL/NC scaffolds, PCL nanofibers containing 4% NC exhibited good viability along with improved protein adsorption and uniform fiber diameter. While the PCL/6%NC showed less viability and protein adsorption than PCL/4%NC. Thus, PCL/4% NC was found to exhibit the most balanced properties to meet all the required specifications for bone tissue engineering and hence selected for our cell culture experiments. PCL/4%NC will be denoted as PCL/NC hereafter.

4.2.11 Cell attachment and spreading

The difference in hMSC morphology and spreading to the PCL/NC nanofibrous scaffold at different time intervals (4h, 24h and 48h) were viewed under SEM which is shown in Fig.9. Initial cell attachment was observed by fixing the sample immediately after 4h of seeding. The cells appeared somewhat rounded (Fig.5.11b & c) but well attached. By 24h, hMSCs had more elongated morphology on the PCL/NC nanofibers (Fig. 4.2.11d, e, f), indicating adhesion and spreading process on PCL/NC containing scaffolds. Furthermore, extensive spreading and flattening of hMSCs was observed after 48h (Fig. 4.2.11g, h & i). Therefore nanofibers containing NC were found to have good affinity for cells, supporting hMSC adsorption and spreading.
Figure 4.2.11 Attachment of hMSCs on PCL/NC scaffolds: SEM images showing a, b and c showing initial cell attachment after 4h, images d, e, f showing 24h spreading and g, h and I, showing 48h spreading of cells.

The morphologies of hMSCs on nanofibers are projected in Fig. 4.2.12 by fluorescence staining of actin fibers in red. This was taken at two different time points, day 2 and day 4 on both PCL and PCL/NC scaffolds. Upon staining, most of the actin filaments concentrated on the periphery of the cells, which confirmed that the hMSCs were nicely spread on the scaffold surface. On day 2, after cell seeding the hMSC on the PCL/NC nanofibers had well-organized parallel arrays of actin filaments (Fig. 4.2.12c), whereas on bare PCL substrate the actin is much less organized (Fig. 4.2.12a) indicating enhanced adhesion and spreading process on NC containing scaffolds. Even after 4 days of culturing, the cells were not yet fully spread on PCL scaffolds (Fig. 4.2.12b). In contrast to that, hMSCs formed a more defined cytoskeletal arrangement on PCL/NC scaffolds (Fig. 4.2.12d).
4.2.12 Cell Proliferation

Fig. 4.2.13 demonstrates the proliferation of hMSCs and their retention on nanofibrous scaffolds over a period of 6 days, estimated quantitatively using alamar Blue assay. Compared to PCL control scaffolds, cell number is significantly higher on PCL/NC scaffold at every time point from 1 to 6 days (Fig. 4.2.13a), although approximately same number of cells were seeded on both the scaffolds. The cells grew robustly on PCL/NC scaffolds than on PCL substrate which was further substantiated by DAPI staining of cell nuclei on scaffolds PCL (Fig. 4.2.13 b, c) and PCL/NC (Fig. 4.2.13 d, e). Thus the inclusion of NC into electrospun PCL nanofibers proved favorable for cell attachment and has advantage over pure PCL scaffolds.
Figure 4.2.13 Proliferation of hMSCs on PCL and PCL/NC scaffolds: a) Graph showing hMSC proliferation at various time points from day 1 to day 6 illustrating increase in cell number over time (* level of significance $p \leq 0.05$). Fluorescent images showing nuclei of hMSCs stained with DAPI on b) PCL & c) PCL/4%NC after 24h and after 72h on d) PCL & e) PCL/4%NC.

4.2.13 Differentiation studies of hMSCs on scaffolds

ALP is one of the main components of bone matrix vesicles and it is an early indicator of immature osteoblast activity\textsuperscript{138}. ALP activity was measured as a marker of differentiation of hMSCs and also of osteoblastic phenotype expression. The amount of ALP present was evaluated (Fig. 4.2.14) in hMSCs stimulated with osteogenic medium with respect to unstimulated cells cultured in normal medium alone. On day 1 after osteogenic induction, ALP concentration was slightly increased on control PCL scaffolds in OM with respect to the PCL/NC scaffolds. But after one week, there was a sudden rise in ALP concentration in PCL/NC scaffolds treated in OM and a very little increase in ALP level in control scaffolds treated in OM. On further culturing for one more week, ALP activity started reducing in PCL/NC cell constructs while it was still increasing in PCL control scaffolds. This
demonstrates that NC containing scaffolds stimulates early differentiation of hMSC into osteoblast much more rapidly than bare PCL scaffolds.

Figure 4.2.14 ALP activity assay on scaffolds: Alkaline phosphatase activity of hMSCs with and without osteogenic medium, upon induction from day 1 to day 21 in culture.

NC is a silicate and hence predicted to be osteoinductive, as silica is osteogenic agent\textsuperscript{139}. Among the scaffolds treated in normal medium, PCL/NC showed higher ALP activity than PCL thereby supporting the fact that NC has the ability to induce differentiation even without osteogenic stimuli, but to a small extent.

Also the \textit{RUNX2} protein was expressed much earlier on the PCL/NC scaffolds i.e day 7(Fig. 4.2.15d), compared to native PCL which proves that the osteogenic potential was higher on the scaffolds containing intercalated NC.
Immunocytochemistry showed the presence of *RUNX2* localized within the nuclei of the hMSCs on PCL/NC scaffolds, at Day 7, 14 and 21 (Fig.4.2.15) in osteogenic medium. This early expression of *RUNX2*, a gene expressed in pre-osteoblasts is a mark of early induction of
differentiation of hMSCs on the PCL/NC nanocomposite scaffolds compared to PCL scaffolds. This is the first study that suggests that hMSCs may differentiate more readily when exposed to embedded nanoclay in PCL nanofibrous scaffolds. Thus the osteogenic potential of the scaffold is enhanced on nanoclay incorporation.

4.3 MR-Functional Nano-Hydroxyapatite Incorporated PCL Composite Scaffolds for In situ Monitoring of Bone Tissue Regeneration by Magnetic Resonance Imaging

In this part of the thesis, we looked into a step forward in tissue engineering, i.e monitoring the tissue regeneration. Towards achieving this we incorporated the MF-nHAp particle which has multifunctionality, one of them being MR-functionality as mentioned earlier.

4.3.1 Research question

- Will the scaffold containing the MF-nHAp be biocompatible and if so, will it enhance the osteogenic potential of the polymeric scaffold?
- What are the contrast changes observed during the process of bone tissue regeneration using the composite containing the MR functional bioceramic when monitored by MRI?

4.3.2 Hypothesis

- The scaffold containing MF-nHAp will be biocompatible and would enhance the osteogenic potential of the scaffold.
- The stages of bone tissue regeneration of the composite containing MR functional bioceramic, along with hMSCs can be monitored by MRI.

4.3.3 Characterization of PCL/nHAp and PCL/MF-nHAp nanocomposite fibrous scaffold

SEM images in figure 4.3.1 show the electrospun nanofibrous scaffolds of PCL, PCL/nHAp and PCL/MF-nHAp. The obtained nanofibers were randomly oriented and were in the range of 100-500nm. The incorporation of the HAp and MF-nHAp particles delivered fibers of diameters very similar to that of native PCL (Fig. 4.3.1C). These nanofibrous structures were very similar to the earlier composite scaffolds.
Figure 4.3.1  **Electrospun nanofibrous scaffolds:** Scanning electron micrographs showing (A) PCL (B) PCL/nHAp and (C) PCL/MF-nHAp electrospun nanofibrous scaffolds.

4.3.4  **Contact Angle Measurement**

The contact angle was measured with milli-Q water in order to check the hydrophilicity/hydrophobicity of the scaffold. PCL on its own showed a contact angle of 146.2 ± 0.6° (Table 4.3.1.), whereas the fabricated composite scaffolds showed a smaller contact angle, namely, 137.5 ± 1.2° and 130.8 ± 1.0° respectively for PCL/nHAp and PCL/MF-nHAp (Table 4.3.1.). The measured contact angle value reflected the decreased hydrophobicity of the composite scaffolds compared to PCL.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Contact angle (θ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCL</td>
<td>146.2 ± 0.6</td>
</tr>
<tr>
<td>PCL/nHAp</td>
<td>137.5 ± 1.2</td>
</tr>
<tr>
<td>PCL/MF-nHAp</td>
<td>130.8 ± 1.0</td>
</tr>
</tbody>
</table>

The decrease of the contact angle (Table 4.3.1) by HAp addition to PCL is consistent with the fact that HAp itself is relatively hydrophilic with a contact angle of approximately 65.2 with water as reported earlier compared to about 146 for PCL alone. A study by Chuenjikutaworn et al demonstrated that PLLA/HAp composite fiber mats had a lower contact angle compared to PLLA fiber mat and that the HAp particles on the surface of the fiber mats causes the scaffold to be hydrophilic. Similarly a study on PLGA/nHAp scaffolds also showed that incorporation of nHAp makes the scaffold to be hydrophilic.
4.3.5 Mechanical testing

Tensile stress at failure of electrospun native PCL scaffold was found to be 1.3 MPa, and that of PCL/nHAp was found to be 1.7 MPa (Fig. 4.3.2). Stress at failure of PCL/MF-nHAp scaffold was as high as 3.35 MPa. Hence the composite scaffolds showed higher mechanical strength compared to native PCL.

![Graph showing tensile strength of the three electrospun nano-composite scaffolds, namely PCL, PCL/nHAp and PCL/MF-nHAp (* shows significance compared to PCL, with p ≤ 0.05)](image)

**Figure 4.3.2 Mechanical testing of the nano-composite scaffolds.** Graph showing tensile strength of the three electrospun nano-composite scaffolds, namely PCL, PCL/nHAp and PCL/MF-nHAp (* shows significance compared to PCL, with p ≤ 0.05)

Our previous work reported the PCL/nHAp nanofibrous scaffold to be mechanically stronger compared to nanofibrous native PCL scaffolds. The mechanical strength of the baseline PCL/nHAp used as a control in this study was also found to be stronger than PCL consistent with the previous study. The increase in mechanical strength could be attributed to the nHAp which is known to increase the stiffness and compressive strength of native bone. What is unique here is the role of MF-nHAp on strength. As Fig.4.3.2 showed, the strength increased by about 153% with addition of 2.63 wt.% MF HAp compared to only 30% increase when the same amount of conventional nHAp was added. This higher strength may be attributed to the smaller PCL diameter (Fig. 4.3.2) in the PCL/MF-nHAp composite scaffold. It is expected that for a given volume of fiber deposited the fiber spacing would scale linearly with the fiber diameter, hence the pore sizes would also decrease which can improve strength. These morphological changes induced by MF-nHAp may be the causative factor in the strength increase. The small amounts of doped Gd (6%), is not expected to change the
mechanical properties of nHAp but it could influence the nHAp/PCL interface characteristics by providing additional positively charged sites on the surface for interaction with the polymer which can in turn influence strength. It is well known that polymers interact with HAp by binding to its positively charged sites\textsuperscript{145}. The size of the MF nHAp and nHAp are similar as was reported earlier\textsuperscript{91} by us, hence this factor could not have played a role in the strength increase.

### 4.3.6 Leaching studies of gadolinium from the scaffolds

ICP-AES analysis detected only a very small amount of gadolinium in the medium containing the PCL/MF-nHAp scaffold. The spectroscopy results showed 0.2 ppm of gadolinium on day 1, 0.23 ppm on day 2 and 0.27 ppm on day 4 (Table 4.3.2). The other two scaffolds, PCL and PCL/nHAp did not show any presence of gadolinium as expected.

#### Table 4.3.2 ICP-AES analysis after incubating scaffolds in medium:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Time Interval (No. of Days)</th>
<th>Gd (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCL</td>
<td>1</td>
<td>BDL</td>
</tr>
<tr>
<td>PCL/nHAp</td>
<td>1</td>
<td>BDL</td>
</tr>
<tr>
<td>PCL/MF-nHAp</td>
<td>1</td>
<td>0.2</td>
</tr>
<tr>
<td>PCL</td>
<td>2</td>
<td>BDL</td>
</tr>
<tr>
<td>PCL/nHAp</td>
<td>2</td>
<td>BDL</td>
</tr>
<tr>
<td>PCL/MF-nHAp</td>
<td>2</td>
<td>0.23</td>
</tr>
<tr>
<td>PCL</td>
<td>4</td>
<td>BDL</td>
</tr>
<tr>
<td>PCL/nHAp</td>
<td>4</td>
<td>BDL</td>
</tr>
<tr>
<td>PCL/MF-nHAp</td>
<td>4</td>
<td>0.27</td>
</tr>
</tbody>
</table>

BDL - below detectable limit (i.e. 0.1 ppm)

This is well below the safe levels of gadolinium which is 2 ppm\textsuperscript{146}. Hence the amount of gadolinium which leached out from the MF-nHAp even after 4 days is well within allowable limits.
4.3.7 Protein adsorption studies

The protein adsorption of the composite scaffolds incubated in culture media at different time intervals were assessed by BCA assay. The incorporation of nHAp and MF-nHAp into the scaffolds significantly improved the amount of protein adsorbed after 8h on PCL/nHAp and PCL/MF-nHAp scaffolds. Values were 9.5 µg/mg scaffold in PCL/nHAp and 11.6 µg/mg scaffold in PCL/MF-nHAp scaffolds compared to PCL which was 8.9 µg/mg scaffold (Fig. 4.3.3).

![Graph showing protein adsorption on nanofibrous scaffolds](image)

**Figure 4.3.3 Protein adsorption on nanofibrous scaffolds:** Graph shows the total protein adsorbed on PCL/nHAp and PCL/MF-nHAp scaffolds compared to PCL nanofibers within 2-6h duration. Values represent mean ± S.D of three independent experiments. (*shows significance compared to PCL, with p ≤ 0.05)

As shown in figure 4.3.3, the protein adsorption increased due to nHAp additions in the nanofibrous scaffolds. This is consistent with several other studies\(^{144, 147, 148}\). Ma et. al.\(^{149}\) have suggested that the irregular surfaces of the nHAp can contribute to enhanced protein adsorption.

Webster and colleagues\(^{150, 151}\) have shown significant increase in protein adsorption on nanosized ceramic surface compared to micron sized ceramic surface due to increase in surface area. This is also consistent with the present results wherein nanosized HAp particles were added to the PCL.
4.3.8  *In vitro* mineralization studies of scaffolds

Mineralization studies showed that on 21st day after incubating in SBF, there were mineral deposits on both the composite scaffolds (Fig. 4.3.4A & 4.3.4C). The Ca/P ratios of the mineral deposits on PCL/nHAp and PCL/MF-nHAp scaffolds were found to be 1.802 and 1.9 respectively (Fig. 4.3.4B & 4.3.4 D). The Ca/P ratios of the nHAp and MF-nHAp were ≈ 1.65.

![Figure 4.3.4](image)

**Figure 4.3.4** SEM images and EDS graphs of mineralized composite scaffolds: SEM images of (A) PCL/nHAp and (C) PCL/MF-nHAp mineralized scaffolds after 21 days in SBF. EDS graphs of (B) PCL/nHAp and (D) PCL/MF-nHAp mineralized scaffolds after 21 days in SBF showing the Ca/P ratios.

The mineralization studies conducted on the scaffolds showed that there were mineral deposits on both the PCL/nHAp as well as the PCL/MF-nHAp scaffolds. The Ca/P ratios of the mineral deposits that formed on both the composite scaffolds were found to be ≈ 1.9 (Fig. 4.3.4). This could be due to more deposition of tetracalcium phosphate minerals. In a previous study\(^{152}\), tetracalcium phosphate was proved to have bioresorption behavior. On the other hand, the Ca/P ratio of the incorporated nHAp particles by themselves was approximately 1.65 with the values for MF-nHAp slightly lower than for nHAp. Thus the Ca/P ratio for MF-nHAp is closer to that for native bone, namely, 1.631 \(^{153}\). An earlier
study\textsuperscript{154} showed that the incorporation of nHAp could promote and improve the deposition of bone-like apatite crystals on the porous composite scaffolds. Improvement in the amount of deposition, and also uniformity of the apatite particle deposition was shown on nHAp/PLLA composite scaffolds, than on pure PLLA scaffolds.

4.3.9 Cell viability by alamarBlue on scaffolds

The cytocompatibility of the composite scaffolds were checked by direct contact method. Alamar Blue assay results at 48h show that the PCL/nHAp and PCL/MF-nHAp scaffolds sustain 98 and 120% viability (Fig. 4.3.5A) of hMSCs compared to PCL scaffolds respectively. Both the composite scaffolds were thus found to be biocompatible. The PCL/MF-nHAp scaffolds showed significant rise in the viability at 48, 96 and 144 h time points (Fig.4.3.5B). On day 1 scaffolds show 10,000 cells per scaffold and on Day 6 number of cells rise to approximately 82,000 on PCL/MF-nHAp compared to 60,000 on PCL/nHAp. The rate of proliferation of hMSCs on the PCL and PCL/nHAp scaffolds we found to be similar (Fig. 4.3.5B).
Figure 4.3.5 Cell viability and proliferation studies on scaffolds by alamarBlue: (A) Cell viability of hMSCs on scaffolds compared to their respective controls after 48h and (B) Proliferation of hMSCs for a period of 1-6 days. Values represent mean ± S.D of three independent experiments. (*shows significance compared to PCL, with p ≤ 0.05)

4.3.10 Proliferation studies

The alamarBlue assay results show that the PCL/nHAp scaffolds show higher rate of proliferation compared to PCL scaffolds. To confirm whether the PCL/MF-nHAp scaffolds support proliferation, MTS assay and PicoGreen assay were done. The PCL/MF-nHAp scaffolds showed significantly higher rate of proliferation compared to PCL scaffolds by both MTS assay as well as DNA quantification assay, as shown in figure 4.3.6. Apart from this
the protein Ki-67 was also expressed in more number of cells on PCL-MF-nHAp scaffolds compared to PCL scaffolds (Fig. 4.3.6C & D).

Figure 4.3.6  Proliferation of hMSCs on scaffolds: Graphs showing (A) proliferation by MTS assay and (B) DNA quantification from hMSCs on scaffolds at 1-6days. Fluorescent images of hMSCs on (C) PCL and (D) PCL/MF-nHAp scaffolds, stained for Ki-67 protein (yellow) and nuclei (red). Values represent mean ± S.D of three independent experiments. (*shows significance compared to PCL, with p ≤ 0.05)

The PCL/MF-nHAp scaffolds showed a significant increase in proliferation (Fig.4.3.6) compared to the corresponding nHAp containing scaffolds. This could be due to the presence of the gadolinium in the nanofiller. As shown in earlier studies, 2-60µM gadolinium was found to promote proliferation in cells. The gadolinium could promote the cell cycle progression by enhanced entry to S-phase by activating ERK and PI3K signaling pathways, which are essential regulators of cell cycle progression. Fu et al. showed by addition of gadolinium to the cells, promoted to cell cycle progression, where the particles could have entered the cells or mere physical adsorption might have enhanced the proliferation. In our study, 0.2-0.27ppm of gadolinium was being leached out from the
PCL/MF-nHAp scaffolds (Table 4.3.2) which could be acting on proliferation of cells along similar lines. The exact mechanism behind this is yet to be found. Here the small concentration of 0.01nM gadolinium doping present in PCL/MF-nHAp was able to induce proliferation in hMSCs (Fig. 4.3.6).

4.3.11 Cell attachment and spreading on scaffolds

Cell adhesion studies showed that the hMSCs were attached and well spread on the scaffolds after 12h in culture (Fig.4.3.7A, B & C). In both the PCL/nHAp and PCL/MF-nHAp nano-composite scaffolds the morphology of the cells were flattened and elongated. These results did not appear to alter by the presence or absence of nHAp in the fibers. The morphologies of hMSCs on composite nanofibers at 24h are projected in figure 4.3.7D, E & F by fluorescence staining of actin fibers in red and nuclei in blue and the nanofibers in green. The extended actin filaments show the well spread cells on the nanocomposite scaffolds. The staining shows that there is fine spreading of cells on PCL/MF-nHAp scaffolds.

Figure 4.3.7 hMSC attachment and spreading on scaffolds: SEM images of hMSCs attached on A) PCL B) PCL/nHAp and C) PCL/MF-nHAp scaffolds after 12h of incubation. Confocal images of hMSCs after 24h on D) PCL, E) PCL/nHAp and F) PCL/MF-nHAp scaffolds stained for actin (red), DAPI (blue) and nanofibrous scaffolds (green).
4.3.12 Differentiation of hMSCs on nanocomposite scaffolds

Differentiation of hMSCs, measured by quantifying the amount of ALP enzyme at various time points was as plotted in figure 5.8 for various scaffolds (PCL alone and PCL with MF-nHAp) in both normal and osteogenic medium. ALP activity on all scaffolds was below 1µg/mg protein on day1. On day 7 and day 14, the ALP activity rose to 2-4µg/mg protein on nanofibrous scaffolds in osteogenic medium. On day 21, the ALP activity was significantly high in hMSCs on the scaffolds in osteogenic medium, while on the scaffolds in normal medium remained low. The hMSCs on PCL/MF-nHAp scaffolds showed a significant increase in ALP activity (Fig. 4.3.8) compared to PCL scaffolds on Day 21. The protein expression of RUNX2 was expressed on Day 7 on nHAp and MF-nHAp containing scaffolds, while on native PCL expression was found only on Day 14 (Fig. 4.3.9). All scaffolds showed RUNX2 expression on days 14 and 21.

![Figure 4.3.8](image)

**Figure 4.3.8** The alkaline phosphatase activity in hMSCs on composite scaffolds: ALP activity of hMSCs on PCL, PCL/nHAp and PCL/MF-nHAp scaffolds in normal medium(NM) and osteogenic medium(OM) over time (1-21days). Values represent mean ± SD and (* shows significance compared to PCL, with p ≤ 0.05).

Both ALP activity and RUNX2 expression were checked to confirm differentiation of hMSCs. The concentration of the ALP enzyme was found to be the highest in the PCL/MF-nHAp scaffolds on 21st day in osteogenic medium (Fig. 4.3.8). Also the RUNX2 protein was expressed much earlier on the PCL/nHAp (Fig. 4.3.9E) as well as the PCL/MF-nHAp (Fig. 4.3.9F) scaffolds compared to native PCL (Fig. 4.3.9D), which proves that the osteogenic
potential was higher on the scaffolds containing nHAp\textsuperscript{142, 143} and MF-nHAp nanoparticles. \textit{RUNX2} expression was similar in both the composite scaffolds.

The differentiation studies on scaffolds confirmed that the hMSCs could differentiate to osteoblasts on these scaffolds. The \textit{RUNX2} protein was expressed much earlier on the PCL/nHAp (Fig. 4.3.9E) as well as the PCL/MF-nHAp (Fig. 4.3.9F) scaffolds compared to native PCL (Fig. 4.3.9D), which is consistent with the results seen in previous studies with

Figure 4.3.9 Immunocytochemistry of \textit{RUNX2} in hMSCs on scaffolds: Fluorescent images (merged) showing hMSCs on (A, B & C) Day 1, (D, E & F) Day 7, (G, H & I) Day 14 and (J, K & L) Day 21 in osteogenic medium on scaffolds PCL, PCL/nHAp and PCL/MF-nHAp scaffolds respectively, stained for \textit{RUNX2} (red), DAPI (blue) and nanofibrous scaffolds (green).
nHAp\textsuperscript{157,158}. This proves that the osteogenic potential of hMSCs was higher on the scaffolds containing nHAp\textsuperscript{159, 160} and MF-nHAp nanoparticles. ALP activity is found in both pre-osteoblasts as well as osteoblasts. The ALP activity measured in this study, had a similar trend to that seen in our previous study\textsuperscript{161}, which showed enhanced osteogenic potential of hMSCs on composite nanofibrous scaffolds. The hMSCs had significantly higher osteogenic potential on MF-nHAp containing scaffolds. This is the first study that suggests that hMSCs may differentiate more readily when exposed to MF-nHAp within the PCL nanofibers.

### 4.3.13 MRI by T1 weighted contrast of the scaffolds

The $T_1$ weighted images of PCL scaffold with and without hMSCs were taken as the control samples. PCL/nHAp scaffolds showed no contrast changes compared to control over a period of 21 days. The PCL/MF-nHAp scaffold without cells showed a bright contrast compared to native PCL scaffold (Fig.4.3.10). This bright contrast of the scaffold got even brighter after 7 days of hMSCs cultured on them in osteogenic medium. On 14\textsuperscript{th} day after culture of hMSCs the contrast was the brightest. After the 21\textsuperscript{st} day in differentiating medium, the hMSCs started differentiating and the contrast of the PCL/MF-nHAp reduced and faded to that of control scaffolds (PCL & PCL-nHAp) (Fig. 4.3.10). The schematic representation of the changes in the scaffold during bone regeneration and the corresponding MRI is given in figure 4.3.12. This pictorially represents the changes in a MR functional scaffold during bone regeneration.

![Figure 4.3.10 MRI by T1 weighted contrast of the scaffolds](image)

**Figure 4.3.10 MRI by T1 weighted contrast of the scaffolds:** The images showing PCL scaffolds (bottom panel), PCL/nHAp scaffolds (middle panel) and PCL/MF-nHAp scaffolds (top panel) with and without hMSCs.
The mean intensities of the samples in MRI were measured in order to get a quantitative value of the brightness and are as shown in figure 4.3.11. The intensity values also show that the brightness of the PCL-MF-nHAp scaffold grows after 7 and 14 days of hMSCs in osteogenic medium. The intensity reduced by day 21. This correlates to the bone regeneration taking place.

![Changes in intensity during tissue regeneration](image)

**Figure 4.3.11 Intensities of the scaffolds in MRI image:** Graph showing the mean intensities of MR contrast obtained of PCL/nHAp and PCL/MF-nHAp scaffolds compared to PCL at various time points.

The MF-nHAp particles were doped with Gd$^{3+}$, which is paramagnetic and possess high magnetic moment due to isotropic electronic ground state $^8S_{7/2}$ and half-filled f-orbital. This leads to large effects on both longitudinal and transverse proton relaxivities of Gd$^{3+}$ even at low applied magnetic fields$^{162}$. The scaffold containing this MF-nHAp showed contrast changes by MRI over the regeneration period (Fig.4.3.10 & 4.3.11). The changes taking place in an implant during in vitro bone regeneration is schematically shown in figure 4.3.12. Here, we also show the hypothetical change in MR contrast at various stages.
Figure 4.3.12 Schematic diagram of the contrast changes by MRI during bone regeneration in vitro: The picture shows the different stages of bone regeneration and the corresponding changes in the contrast MRI, when PCL/MF-nHAp scaffold is being monitored.

The native PCL scaffold is hydrophobic and hence may not enable effective proton exchange with the physiological liquid around the scaffold which is critical for bright MR contrast. In the MF-nHAp incorporated PCL sample without cells, the hydrophobicity of the polymer rendered poor proton exchange between contrast agent and water (Fig.4.3.10). When cells start growing and proliferating on these scaffolds the contrast increase rendered a bright image by day 7 and day 14. This indicates that when the cells are in contact with the scaffold, the hydrophobic polymer interacts better with the lipid content\textsuperscript{163} of the cell membrane and thereby facilitating a better proton exchange with the nano contrast agent (MF-nHAp). Interestingly the MR contrast of scaffolds peaked at day 14 and decreased (Fig.4.3.11) as minerals started to deposit and tissue started developing\textsuperscript{164, 165}, by day 21. The clear implication of this is that the contrast is dependent on the stage of regeneration of the tissue and hence this provides the possibility of monitoring tissue regeneration. The present composite provides this useful diagnostic capability while at the same time maintaining the excellent bone forming capacity and strength of the bone.
4.4 Comparison of the three different composite PCL nanofibrous scaffolds with PCL

This section intends to give a clear picture about the different nanofillers on incorporation in PCL nanofibrous scaffolds. It is a comparative study between these PCL/nanofiller composite scaffolds for assessing their regenerative potential for the purpose of bone tissue engineering. We here compare the mechanical strength, contact angle, protein adsorption, biocompatibility of hMSCs, their attachment and differentiation to osteoblasts on the various scaffolds.

4.4.1 Mechanical strength

The PCL/MF-nHAp scaffolds showed highest mechanical strength 158% increase in strength compared to the PCL scaffold. PCL/nSiO₂ scaffold was found to be mechanically strong with 120% fold increase compared to native PCL scaffold. The PCL/NC scaffolds showed 110% fold increase in strength compared to the PCL scaffold. There was significant difference between group means as determined by one-way ANOVA (F= 180.91, p ≤ 0.001)

4.4.2 Contact angle

The contact angle of PCL/8% nSiO₂ scaffold was found to be the least 118.2 ± 0.2. Hence this was the scaffold which was least hydrophobic compared to all scaffolds. There was significant difference between group means as determined by one-way ANOVA (F= 396.94, p ≤ 0.001)

4.4.3 Cell Proliferation

Proliferation studies clearly show that PCL/MF-nHAp scaffolds promote proliferation of hMSCs. There is a 130% increase in proliferation compared to PCL nanofibrous scaffolds. Apart from this, none of the scaffolds studied, showed this increased proliferation compared to PCL scaffolds. This could be due to the Gd doping present in the PCL/MF-nHAp scaffolds as discussed earlier. There was significant difference between group means as determined by one-way ANOVA (F= 66.13, p ≤ 0.001)
4.4.4 Protein adsorption

The PCL/nSiO$_2$ scaffolds show the highest increase in the protein adsorbed (i.e. 100-120% with respect to PCL) compared to any other scaffold included in the study (compare Fig. 4.1.12, Fig. 4.2.8 & Fig. 4.3.3). This could be due to the smaller size of nSiO$_2$ particles (10nm) used in the study compared to NC, nHAp and MF-nHAp, leading to a greater increase in surface area per unit weight of nSiO$_2$ in the scaffold, and hence the protein adsorbed. It is reported earlier$^{166}$ that smaller size particles provide higher specific surface area which enhances protein adsorption. PCL/4%NC shows 60% increase of in the amount of protein adsorption compared to native PCL scaffold and PCL/MF-nHAp scaffold 20-40% increase compared to PCL scaffolds.

There was significant difference between group means as determined by one-way ANOVA at different time points are 2h ($F = 82.45$, $p \leq 0.001$), 4h ($F = 12.06$, $p \leq 0.001$), 6h ($F = 237.24$, $p \leq 0.001$) 8h ($F = 88.29$, $p \leq 0.001$).

4.4.5 Osteogenic differentiation

Osteogenic differentiation was was best seen on the PCL/NC scaffolds compared to all the scaffolds studied with a 140-270% increase in ALP activity compared to PCL nanofibrous scaffold. PCL/nSiO$_2$ scaffold showed 100-120% increase in ALP activity and hence enhanced differentiation of hMSCs to osteoblasts in osteogenic medium compared to PCL nanofibrous scaffolds. PCL/Mf-nHAp scaffolds showed of 100-120% increase in ALP activity compared to PCL scaffolds. There was significant difference between group means as determined by one-way ANOVA at different time points were 14$^{th}$ day($F = 31.64$, $p \leq 0.001$) and 21$^{st}$ day ($F = 852.9$, $p \leq 0.001$)

Summarizing the comparative results, the PCL/MF-nHAp scaffold is much more favourable to proliferation of hMSCs and differentiation of hMSCs to osteoblasts. Above all, these scaffolds have MF-nHAp particles incorporated, which impart MR functionality to the scaffolds. These scaffolds may help in monitoring the changes during bone regeneration. Of the three scaffolds, PCL/MF-nHAp is the best scaffold for bone tissue engineering.