CHAPTER 7

PRECIPITATION OF HYDROXYAPATITE ON ELECTROSPUN PCL/ALOE VERA/SILK FIBROIN BIOCOMPOSITE NANOFIBROUS SCAFFOLD FOR ADIPOSE DERIVED STEM CELLS DIFFERENTIATION TO OSTEOBLAST LINEAGE

7.1 INTRODUCTION

Electrospinning is a multipurpose technique which offers possible approach to produce continuous nano and micro dimensional polymeric fibres with large surface areas making this technique very attractive for diverse applications. The major application of this technique in tissue regeneration is co-spinning of polymers with bioactive materials to create biomimetic scaffolds with suitable physical and biological environment to promote cell growth and new tissue formation (Murugan & Ramakrishna 2006, Dhandayuthapani et al 2011, Xie et al 2008, Dehai et al 2007). For bone tissue engineering, scaffold should be biocompatible, osteoconductive, osteoinductive and have appropriate structural and mechanical properties (El-ghannam 2005). Synthetic polymers such as poly lactic acid, poly glycolic acid, PCL and natural polymers such as chitosan, gelatin, collagen, fibrin and hyaluronic acid have been enormously used for soft tissue regeneration and as drug delivery systems (Silvia et al 2012), but their application in hard tissue surgery has raised several problems such as inflammatory reaction. This
drawback can be significantly overcome by the incorporation of native biomaterials, such as HA, SF and AV as justified in chapter 6.

Stem cells in addition to biomimetic scaffolds are required in bringing tissue regeneration. Adipose tissues are abundant source of adult multipotent stem cells capable of undergoing multilineage differentiation. Adipose derived stem cells (ADSC) have similar surface immunophenotype, and osteogenic differentiation like bone marrow derived stem cells. Abundance, easy access and lower cytotoxicity make ADSC a better alternative to bone marrow stem cells, in conditions where large number of cells is required for regeneration (Nnodim 1987, Deslex et al 1987, Gimble et al 2007, Liu et al 2007). The current study focuses on the fabrication of nanofibrous scaffold using PCL with incorporation of natural biomaterials AV for osteoinduction and SF for mechanical support with surface mineralized HA to prevent immune response and the scaffolds developed were investigated, in terms of the initial cell adhesion, proliferation and further osteogenic differentiation and mineralization of ADSC cells for bone tissue engineering.

7.2 MATERIALS AND METHODS

7.2.1 Materials

Adipose derived stem cells were obtained from Lonza, USA. Dulbecco’s modified eagle’s medium/Nutrient Mixture F-12 (HAM), fetal bovine serum (FBS), antibiotics and trypsin-EDTA were purchased from GIBCO Invitrogen USA. CellTiter 96® Aqueous one solution was purchased from Promega, Madison, WI, USA. PCL, 1, 1, 1, 3, 3, 3-hexafluoro-2-propanol (HFP), CaCl₂, Na₂HPO₄, Alizarin Red-S and Cetylpyridinium chloride were purchased from Sigma. Lyophilized Aloe vera and silk fibroin
powder were purchased from Xi’an Yuensun Biological Technology Co., Ltd, China.

7.2.2 Fabrication of Nanofibrous Scaffolds

PCL pellets were dissolved in HFP at 10% (w/v) and PCL-AV-SF solution was prepared at the ratio of 8:4:4 at the concentration of 16% (w/v) in HFP. The solutions were magnetically stirred at room temperature overnight for better distribution and homogenization. The polymer solution was then loaded into a 3 ml standard syringe attached to 21G blunt needle using a syringe pump (KD 100 Scientific Inc., Holliston, MA., US) at a constant flow rate of 1 ml/h with a voltage electric field of 17 kV (DC high voltage power supply from Gamma high voltage research, Florida, USA). The electrospun nanofibers were collected on aluminum foil wrapped on a flat collector plate kept at a distance of 10 cm between the tip of the spinneret and collector plate for physico-chemical analysis. Nanofibers were collected on 15 mm coverslips for cell culture experiments. Fabricated electrospun nanofibrous scaffolds were consequently dried overnight under vacuum oven to eliminate residual solvents. Biomineralization procedure was then carried out on the PCL-AV-SF samples to precipitate HA by calcium-phosphate dipping method. The electrospun PCL-AV-SF nanofibers were initially immersed in 0.5 M CaCl$_2$ solution for 10 min. The samples were then rinsed in DI water for 1 min. The samples were then immersed in 0.3 M of Na$_2$HPO$_4$ for 10 min and rinsed for 1 min in DI water. This entire procedure was considered as one cycle. The scaffolds were subjected to three cycles of the above treatment. The first cycle was for 10 min and the subsequent cycles were for 5 min in each solution. After biomineralization the scaffolds were dried overnight for further studies.
7.2.3 Characterization of Nanofibrous Scaffolds

The physico-chemical characterization of PCL, PCL-AV-SF and PCL-AV-SF-HA nanofibrous scaffolds were analysed by FESEM, FTIR, water contact angle and tensile tester as described in section 3.2.2 under chapter 3.

7.2.4 Cell Culture

Adipose derived stem cells (ADSCs) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) with 1% (v/v) antibiotics in a 75 cm$^2$ cell culture flask. The ADSCs were incubated at 37ºC humidified atmosphere containing 5% CO$_2$ for 7 days and the culture medium was changed once in every 3 days. The cultured cells (passage 4) were trypsinized by trypsin-EDTA and replated after counting with trypan blue using hemocytometer. The electrospun nanofibers were collected on coverslips of 15 mm diameter were placed in a 24 well plate with a stainless steel ring to prevent lifting of nanofibers. The fibers were sterilized under UV light for 3 h and the scaffolds were again sterilized with 70% (v/v) ethanol for 30 min and washed thrice with phosphate buffered saline (PBS) for 15 min each in order to remove any residual solvent and subsequently immersed in complete medium overnight before cell seeding. The ADSCs were seeded on the electrospun nanofibers at a cell density of 10,000 cells/well and TCP was used as a control.

7.2.5 Cell Proliferation

The cell proliferation of ADSCs on TCP, PCL, PCL-AV-SF and PCL-AV-SF-HA nanofibrous scaffolds were determined using the colorimetric MTS assay for a period of 7, 14 and 21 days as described in section 6.2.5 under chapter 6.
7.2.6  Cell Morphology

The cell morphology of ADSCs were analysed using after 21 days of seeding cells on TCP, PCL, PCL-AV-SF and PCL-AV-SF-HA nanofibrous scaffolds, as described in section 6.2.6 under chapter 6.

7.2.7  Alkaline Phosphatase (ALP) Activity

ALP activity of ADSCs differentiation into osteogenic lineages was measured using Alkaline Phosphate Yellow Liquid substrate system for a period of 7, 14 and 21 days as described in section 6.2.7 under chapter 6.

7.2.8  Mineralization of Osteogenic Cells

Alizarin Red-S (ARS) is a dye that binds selectively calcium salts and is widely used for mineral staining. On day 14, the scaffolds seeded with ADSCs were subjected to ARS staining following the similar procedure as described in section 6.2.8 under chapter 6.

7.2.9  Immunofluorescent Staining for Osteocalcin

Osteogenic differentiation of ADSCs was confirmed using immunofluorescent staining by employing both ADSCs specific marker protein CD 105 and osteoblast specific marker protein osteocalcin (OCN). The cells on day 21 were primarily fixed with 100% (v/v) ice cold methanol for 15 min. After fixing, the scaffolds were washed with PBS once for 15 min and incubated in 0.5% Triton X-100 solution in PBS for 5 min to permeabilize the cell membrane. Non-specific sites were blocked by incubating the cells in 3% (w/v) BSA (Bovine serum albumin) for 1 h. Primary antibody ADSCs specific marker protein CD 105 (abcam, USA) was added in the dilution (1:100) for 90 min at room temperature. This was followed by adding secondary antibody Alexa Fluor 594 (Invitrogen-Red) in
the dilution 1:250 for 60 min. The scaffolds were washed thrice with PBS and then incubated with osteoblast specific protein osteocalcin (Sigma) in the dilution 1:100 for 90 min. Further secondary antibody Alexa Fluor 488 (Invitrogen-Green) was added in the dilution 1:250 for 60 min. The scaffolds were washed thrice with PBS for 15 min to remove the excess staining. Finally, the cells were incubated with DAPI (4, 6, diamino-2 phenyl indole) (Invitrogen Corp, Carlsbad, CA) in the dilution 1:5000 for 30 min. The samples were removed from 24 well plates and mounted over glass slide using vectashield mounting medium and examined under the fluorescence microscope (Olympus FV 1000).

7.2.10 Statistical Analysis

The data presented are expressed as mean ± standard deviation. Statistical analysis was done using Student’s $t$-test and the significance level of the data was obtained. The $P$-value $\leq 0.05$ was considered to be statistically significant.

7.3 RESULTS AND DISCUSSION

7.3.1 Characterization of Nanofibrous Scaffolds

Nanostructured scaffolds are favourable platforms to recapitulate the organization of natural extracellular matrix for functional bone tissues formation, because nanotopography provides a closer approximation to native bone architecture (McMahon et al 2013). Polymeric fibers developed should be in nano dimensions for providing favourable structural support in the formation of functional tissues.
Figure 7.1 FESEM micrographs of a) PCL, b) PCL-AV-SF and c) PCL-AV-SF-HA nanofibrous scaffolds

Figure 7.1(a-c) shows the FESEM micrographs of PCL, PCL-AV-SF and PCL-AV-SF-HA nanofibrous scaffolds. The average fiber diameters of PCL and PCL-AV-SF nanofibers calculated using ImageJ analysis software were in the range of 515±19 and 133±28 nm. PCL-AV-SF-HA nanofibrous scaffolds showed densely deposited HA nanoparticles on their surface with a mean fiber diameter of 133±28 nm imparting a rough texture to the scaffolds. Research finding reported that the initial osteoblast attachment and mineralization are favourably enhanced by the rough surface provided by hydroxyapatite (Despina et al 2000). The water contact angle parameter of...
PCL, PCL-AV-SF and PCL-AV-SF-HA nanofibrous scaffolds were analysed and was found that PCL nanofibers were hydrophobic having a contact angle of $133^\circ$. The contact angles obtained for PCL-AV-SF and PCL-AV-SF-HA were $51^\circ$ and $26^\circ$ respectively. The wettability nature of hydroxyapatite and water absorption property of *Aloe vera* has imparted hydrophilicity to the hydrophobic polymeric scaffold thereby improving their cell adhesion property (Kay et al 1964, Rúben et al 2013).

Figure 7.2  Tensile stress-strain curves of PCL, PCL-AV-SF and PCL-AV-SF-HA nanofibrous scaffolds
Figure 7.2 shows the nonlinear stress strain graph of PCL, PCL-AV-SF and PCL-AV-SF-HA nanofibrous scaffolds. Tensile stress for PCL, PCL-AV-SF and PCL-AV-SF-HA nanofibrous scaffolds were 1.54 MPa, 4.80 MPa and 10.89 MPa and can bear a strain of 71 %, 67 % and 70 % respectively. The young’s modulus value for PCL, PCL-AV-SF and PCL-AV-SF-HA nanofibrous scaffolds were 13.67 MPa, 14.60 MPa and 23.49 MPa. Previous studies showed that beta structure of silk fibrin provide outstanding mechanical property which in combination with hydroxyapatite gives excellent compressive modulus (Liu et al 2008). The obtained result with increased tensile stress and tensile modulus values for PCL-AV-SF-HA shows synergistic effect of SF and HA which in turn increases the rigidity of the scaffolds which is very important for clinically useful scaffolds that retain their shape during implantation and ideally bear mechanical loads after implantation especially for bone tissue engineering (Fei et al 2012). The values obtained for nanofibers diameter, contact angle and tensile stress, strain and modulus for PCL, PCL-AV-SF and PCL-AV-SF-HA scaffolds were tabulated in Table 7.1.

Table 7.1  Characterization of PCL, PCL-AV-SF and PCL-AV-SF-HA nanofibers for fiber diameter, water contact angle, and tensile properties

<table>
<thead>
<tr>
<th>Nanofiber construct</th>
<th>Fiber diameter (nm)</th>
<th>Water contact angle (º)</th>
<th>Tensile stress (MPa)</th>
<th>Tensile strain (%)</th>
<th>Young’s modulus (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCL</td>
<td>515±19</td>
<td>133</td>
<td>1.54</td>
<td>71</td>
<td>13.67</td>
</tr>
<tr>
<td>PCL-AV-SF</td>
<td>133±28</td>
<td>51</td>
<td>4.80</td>
<td>67</td>
<td>14.60</td>
</tr>
<tr>
<td>PCL-AV-SF-HA</td>
<td>133±28</td>
<td>26</td>
<td>10.89</td>
<td>70</td>
<td>23.49</td>
</tr>
</tbody>
</table>
Figure 7.3 FTIR spectroscopic analysis of a) PCL b) PCL-AV-SF and c) PCL-AV-SF-HA nanofibrous scaffolds showing characteristic peaks of PCL, AV, SF and HA

The FTIR spectrum of PCL, PCL-AV-SF and PCL-AV-SF-HA scaffolds are shown in Figure 7.3(a-c). The characteristics peaks of PCL namely C=O vibration at 1728 cm$^{-1}$, CH$_2$ band vibrations at 1362,1407 and 1465 cm$^{-1}$, esteric COO vibrations at 1181 and 1238 cm$^{-1}$, O-C vibrations at 1099, 1047 and 961 cm$^{-1}$, CH$_2$ rocking vibration occurring at 729 cm$^{-1}$ are clearly seen in the spectrum (Figure 7.3(a)). The FTIR spectrum of PCL-AV-SF scaffolds is shown in Figure 7.3(b). The characteristic peak of functional groups of components present in Aloe vera namely O-acetyl esters, glucan units, pyranoside ring and mannose are at 1736 cm$^{-1}$, 1032 cm$^{-1}$, 879 cm$^{-1}$ and 811 cm$^{-1}$ respectively. Similarly the secondary structure of silk fibroin consists of the major conformations including random coils and $\beta$-sheet.
Peaks for random coil of Amide I, Amide II and Amide III are seen at around 1650 cm\(^{-1}\), 1540 and 1230 cm\(^{-1}\) respectively. Peaks for \(\beta\)-sheet for Amide I, Amide II and Amide III are seen at around 1620 cm\(^{-1}\), 1520 cm\(^{-1}\) and 1270 cm\(^{-1}\) respectively. These characteristic peaks of *Aloe vera* and silk fibroin are seen in Figure 7.3(b) but slightly broadened due to blending confirming the successful incorporation of AV and SF to the polymeric base. The spectrum (Figure 7.3(c)) of PCL-AV-SF-HA shows the features of spectrum (Figure 7.3(b)) and additionally characteristic peaks of HA namely stretching vibration for PO\(_4^{3-}\) at 1053 cm\(^{-1}\), P-O stretch coupled P-O bend peaks at 573 cm\(^{-1}\) and CO\(_3^{2-}\) group at 1400 cm\(^{-1}\) were obtained similar to bone composition. Thus the physicochemical properties of PCL-AV-SF-HA shows desirable properties in terms of morphology, chemical, hydrophilicity and tensile strength, therefore expected to have better cell adhesion, proliferation and mineralization on this scaffolds for bone tissue engineering.

### 7.3.2 Interaction of Cells and Scaffolds

Initial interactions between cells and scaffolds are an important phenomenon in bringing enhanced proliferations which mainly depends on the physical and chemical composition of the scaffolds (Dietmar 2000). The ability for nanofibrous scaffolds to support adhesion and proliferation of ADSCs on TCP, PCL, PCL-AV-SF and PCL-AV-SF-HA nanofibrous scaffolds on day 7, 14 and 21 was determined by MTS assay as shown in Figure 7.4. The proliferation rate of ADSCs on PCL-AV-SF-HA nanofibrous scaffolds increased by 26.58 % (p ≤ 0.001) on day 14 and about 59.19 % (p ≤ 0.001) on day 21 compared to PCL nanofibrous scaffolds. This is because of the presence of bioactive *Aloe vera*, silk fibroin and hydroxyapatite in PCL-AV-SF-HA nanofibrous scaffolds which imparts favourable factors for efficient cell adhesion. Silk fibroin provides integrin recognition sequence on their surface favourable for osteoblast cell adhesion.
and proliferation (Bini et al 2006). The hydrophilic nature imparted by Aloe vera and rough surface topography provided by HA favoured initial cell adhesion (Nga et al 2012, Marzellus et al 2012) and stimulated increased proliferation in PCL-AV-SF-HA nanofibrous scaffolds compared to PCL scaffold. Similarly PCL-AV-SF-HA nanofibrous scaffolds favoured cell proliferation which almost increased linearly by 10.46 % (p ≤ 0.01) and 12.01 % (p ≤ 0.001) compared to PCL-AV-SF nanofibrous scaffolds on day 7 and 14 due to the presence of precipitated HA on the surface of PCL-AV-SF-HA nanofibrous scaffold which acts as osteoconductive medium and favours ingrowth of cells thereby inducing cell migration, adhesion and growth. This result demonstrates that the unique physical and chemical composition of PCL-AV-SF-HA nanofibrous scaffolds favoured the cell adhesion and proliferation compared to other scaffolds.

![Cell proliferation studies of ADSCs on TCP, PCL, PCL-AV-SF and PCL-AV-SF-HA nanofibrous scaffolds on day 7, 14 and 21. * indicates significant difference of p≤0.01; ** indicates significant difference of p≤0.001](image)

**Figure 7.4** Cell proliferation studies of ADSCs on TCP, PCL, PCL-AV-SF and PCL-AV-SF-HA nanofibrous scaffolds on day 7, 14 and 21. * indicates significant difference of p≤0.01; ** indicates significant difference of p≤0.001
Figure 7.5   FESEM images showing the cell-scaffold interactions on day 21 on a) TCP, b) PCL, c) PCL-AV-SF and d) PCL-AV-SF-HA nanofibrous scaffolds at 5000x magnification

The FESEM images of ADSCs (Figure 7.5(a-d)) showed the cell morphology on TCP, PCL, PCL-AV-SF and PCL-AV-SF-HA nanofibrous scaffolds with the secretion of minerals on the surface of cells on day 21. The initial adhesion, migration and proliferation of cells on the nanofibrous scaffolds were consequently followed by differentiation and mineralization.
The trigger for osteogenic differentiation of ADSCs followed by mineralization is the bony environment (Lu et al 2012). It was seen that cells grown on PCL-AV-SF-HA nanofibrous scaffolds showed densely synthesized mineral on their surface which is due to the presence of HA which provided bone like environment for the mineralization of osteoblasts for bone regeneration. The obtained result clearly proves the ADSC differentiation followed by mineralization process. Precipitated HA is responsible for forming dense layer of minerals on the surface of PCL-AV-SF-HA nanofibrous scaffolds as clearly seen in Figure 7.5(d) compared to other scaffolds proving it to be a potential scaffold for conditions where there is reduction in mineralization process like patients with osteoporosis and inactive osteoblast precursors.

### 7.3.3 Mineralization of Differentiated Osteogenic Cells and Osteocalcin Expression

Pluripotent cells which are undergoing differentiation have a very high elevated level of ALP activity (Radio et al 2006). The osteogenic differentiation of ADSC on different scaffolds can be confirmed by determining the activity of the osteogenic marker ALP. Figure 7.6 shows the ALP activity of cells on TCP, PCL, PCL-AV-SF and PCL-AV-SF-HA nanofibrous scaffolds on days 7, 14 and 21. The ALP activity of cells on PCL-AV-SF-HA was significantly higher (p<0.001) by 35.85 % compared to PCL-AV-SF nanofibrous scaffolds on day 7. Research finding showed that hydroxyapatite in presence of pluripotent cells induce osteogenesis (Hajime et al 1990, Sujatha et al 2012) similarly earlier reports showed acemannan present in *Aloe vera* mediated induction of osteogenesis. PCL-AV-SF-HA showed increased rate of cell ALP activity of about 2.86 %, 198.13 % (p<0.01) and 60.03 % (p<0.01) compared to PCL on day 7, 14 and 21. From
the data it is clear that the ALP activity of cells grown on PCL-AV-SF-HA nanofibrous scaffolds were very high compared to PCL scaffold on all three days confirming the enhanced differentiation of ADSC cells into osteogenic lineage.

![Graph showing ALP activity](image)

**Figure 7.6** Alkaline phosphatase activity showing the osteogenic differentiation of ADSCs on TCP, PCL, PCL-AV-SF and PCL-AV-SF-HA nanofibrous scaffolds on day 7, 14 and 21. * indicates significant difference of $p<0.01$; ** indicates significant difference of $p<0.001$
Figure 7.7  Quantitative analysis of the mineralization by differentiated ADSCs on TCP, PCL, PCL-AV-SF and PCL-AV-SF-HA nanofibrous scaffolds on day 7, 14 and 21. * indicates significant difference of p≤0.01

Mineralization following osteogenic differentiation can be determined quantitatively (Figure 7.7) and qualitatively (Figure 7.8, 7.9) using Alizarin Red Staining methodology. PCL-AV-SF-HA scaffolds (Figure 7.7) showed significantly about 18.83 %, 107.97 % (P≤ 0.01) and 183.60 % higher mineral deposition compared to PCL scaffolds on day 7, 14 and 21 respectively and about 37.42 %, 93.30 % and 57.16 % compared to PCL-AV-SF scaffolds on day 7, 14 and 21. The measured absorbance from PCL-AV-SF-HA scaffolds was increased linearly on day 7, 14 and 21 compared to all other scaffolds. These data shows more ADSCs cells on PCL-AV-SF-HA scaffolds have differentiated leading to mineralization phase to
secrete more ECM. Presence of HA is the major factor responsible for high mineral secretion.

Figure 7.8  Optical microscope images showing the secretion of extracellular matrix by ADSCs using Alizarin red staining on day 14 on a) TCP, b) PCL, c) PCL-AV-SF and d) PCL-AV-SF-HA nanofibrous scaffolds at 10x magnification
Figure 7.9 Optical microscope images showing the secretion of extracellular matrix by ADSCs using Alizarin red staining on day 21 on a) TCP, b) PCL, c) PCL-AV-SF and d) PCL-AV-SF-HA nanofibrous scaffolds at 10x magnification

Optical microscopic images in Figure 7.8 and Figure 7.9 of scaffolds with ARS staining after 14 and 21 days of cell culture supported the above quantitative analysis data where high intensity of red coloured stain confirming the presence of calcium deposition was observed on PCL-AV-SF-HA scaffolds.
Osteocalcin is an osteoblast secreted protein playing major role in bone mineralization. Osteocalcin level is generally correlated with bone mineral density. The expression of osteocalcin can be used as a marker for determining osteoblast activity in bone mineralization (Lumachi et al 2009, Sushma et al 2012). For the present analysis both ADSC specific marker protein and osteocalcin specific marker protein were used to discriminate the undifferentiated ADSC cells and differentiated osteoblast cells. The Figure 7.10(a-d) showed that the cells expressing ADSCs specific marker protein CD 105 (red colour). Figure 7.10(e-h) showed differentiated osteogenic cells.
expressing osteocalcin (green colour) marker protein OCN. Dual expression of both CD 105 and OCN in Figure 7.10(i-l) confirmed the osteogenic differentiation of ADSCs. ADSCs cultured on PCL-AV-SF-HA (Figure 7.10(d, h, l)) scaffolds exhibits the characteristic osteoblastic phenotype and high osteocalcin expression representing complete osteogenic differentiation compared to all other nanofibrous scaffolds. From the observed results we have demonstrated that ADSCs cells on PCL-AV-SF-HA nanofibrous scaffolds undergo a well-defined pathway of cell adhesion, proliferation and differentiation followed by ECM secretion making them excellent scaffolds for bone tissue engineering.

7.4 CONCLUSIONS

The significance of this study is the incorporation of natural biomaterials like silk fibroin and Aloe vera on polymeric electrospun nanofibrous scaffolds with surface precipitation of hydroxyapatite to regulate and improve specific biological functions like adhesion, proliferation and differentiation of ADSC cells for bone tissue engineering. ADSC cells are desirable cell therapeutics for bone repair and regeneration due to their abundance and accessibility which showed osteogenic differentiation, in the absence of an induction medium, with the presence of osteoconductive hydroxyapatite and osteoinductive Aloe vera with silk fibroin providing the necessary mechanical support. This demonstrates the therapeutic potential of ADSCs loaded PCL-AV-SF-HA scaffolds that may benefit patients with reduced numbers of native osteoblast precursors in vivo in conditions like osteoporosis.