CHAPTER 4

ALOE VERA AND SILK FIBROIN INCORPORATED
PLACL BIOCOMPOSITE NANOFIBROUS SCAFFOLD
FOR HUMAN DERMAL FIBROBLAST REGENERATION

4.1 INTRODUCTION

Skin is the largest protective barrier of human body. Skin regenerates itself when subjected to minor injuries however severe damages like full thickness dermal loss require effective clinical treatment failure of which may lead to mortality (Peter 2001). Surgical approaches like auto grafts remains the main treatment for thermal injury related skin loss however severe burn patients lack tissue availability requiring an alternative method of skin replacement (Bishara et al 2005). Emergence of tissue engineering mediated therapy has attracted much attention as it offers a better solution to overcome the drawbacks of current limitation in skin transplantation which focuses on regeneration of neotissues from cells with the support of biomaterials and growth factors (Ikada 2006). The cells, scaffold and growth factor are the three key materials for tissue engineering (Chan & Leong 2008). Scaffolds are artificial structure capable of supporting and providing a native environment for the cell adhesion and proliferation to form tissues (Hetel et al 2011, Lu et al 2013).

Several ways available for scaffold production of which electrospinning remains the predominant choice as it can produce materials with nanoscale properties (Vasita & Katti 2006) mimicking the architecture of
the native extracellular matrix (Wei & Ma 2008). Polymeric scaffold of different origin (natural and synthetic) have been investigated for scaffold development (Li et al 2012, Park et al 2012). Natural materials like collagen, fibrin and chitosan are rich in growth factors and are ideal for promoting skin tissue regeneration but are not mechanically strong when electrospun on the other hand biodegradable synthetic materials such as Polycaprolactone (PCL), Poly (L-lactic acid) (PLLA) are stronger, but lack growth factor for tissue regeneration (Brien 2011, Gunn & Zhang 2010, Agarwal et al 2009). The major challenges in scaffold fabrication for dermal regeneration are the need for both complex functionality and biomechanical stability. The alternative solution for the above issue can be overcome by physical hybridizing of polymers (natural biopolymers or synthetic polymers) for a synergistic actions and then converting them into nanofibers which can impart bioactivity and improved mechanical property to the resulting scaffold.

led to its significant usage in biomedical applications (Inpanya et al 2012). Mannose 6-phosphate and acemannan are the important components responsible for many therapeutic properties of Aloe vera which mediate cell signalling pathway for proliferation of fibroblasts (Stashak & Theoret 2008, Davis et al 1994). It promotes epithelialization and collagen synthesis for effective wound healing (Hamman 2008, Reynolds & Dweck 1999, Krishnan 2006, Davis et al 1989).

The present chapter focuses on development of nanofibrous scaffolds from synthetic biodegradable polymer poly (lactic acid-co-caprolactone) PLACL by incorporating silk fibroin for mechanical stability, Aloe vera for its wound healing property. Physico-chemical and biological characterization of the hybrid scaffolds are investigated for its enhanced ability towards wound healing and tissue regeneration.

4.2 MATERIALS AND METHODS

4.2.1 Fabrication of PLACL, PLACL-SF, PLACL-SF-AV and PLACL/Collagen Scaffolds

PLACL was dissolved in DCM: DMF (3:1) (v/v) (Sigma-Aldrich, St. Louis, USA) to form 8 % (w/v) solution and kept in stirring overnight. PLACL-SF solutions were prepared by dissolving 8 % (w/v) PLACL and 4 % (w/v) lyophilized silk fibroin powder (Xi`an Yuensun Biological Technology Co., Ltd, China) in DCM: DMF (3:1) (v/v). Similarly PLACL-SF-AV solution was prepared by dissolving 8 % (w/v) PLACL followed by addition of 4 % (w/v) silk fibroin powder and 4 % (w/v) Aloe vera powder (Xi`an Yuensun Biological Technology Co., Ltd, China) in DCM:DMF (3:1) (v/v). PLACL/Collagen solution was prepared by dissolving 8 % (w/v) PLACL and 2 % (w/v) collagen in 10 ml HFP. The solutions were fed into a 5 ml standard syringe attached to a 21G blunted stainless steel needle respectively using a
syringe pump (KDS 100, KD Scientific, Holliston, MA) at a flow rate of 0.75 ml/hr with an applied voltage of 17 kV for all the solutions (Gamma High Voltage Research, USA). Random fibers were collected on a flat collector plate wrapped with aluminum foil that was kept at a distance of 12 cm from the needle tip. On application of high voltage the polymer solution was drawn into fibers. These nanofibers were collected on 15 mm cover slips by spreading them on the collector plate and used for cell culture studies.

4.2.2 Characterization of Nanofibrous Scaffolds

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

4.2.3 Culture of Fibroblasts

Human dermal fibroblasts (ATCC, Arlington, VA) were cultured in DMEM (GIBCO Invitrogen, USA) supplemented with 10% (v/v) foetal bovine serum (FBS) (GIBCO Invitrogen, USA) and 1% (v/v) antibiotic/antimycotic solutions (Invitrogen Corp., USA) in a 75 cm² tissue culture flask and incubated at 37°C in a humidified atmosphere with 5% CO₂. The populations of fibroblast passage 4 were used for this experiment. The electrospun scaffolds on coverslips were UV-sterilized, rinsed in phosphate-buffered saline (PBS) and soaked in cell culture medium overnight prior to cell seeding to facilitate protein adsorption and cell attachment. The fibroblasts were separated by trypsinization, centrifuged, counted using a haemocytometer and seeded on the scaffolds at a cell density of 7.5×10³ cells/well and incubated at conditions suitable for cell growth. TCP was used as the control for cell culture studies.
4.2.4 Proliferation of Fibroblasts

The proliferation of cultured human dermal fibroblasts on PLACL, PLACL-SF, PLACL-SF-AV and PLACL/Collagen nanofibrous scaffolds were monitored on the third, sixth and ninth day of culture using the colorimetric MTS assay as described in section 3.2.4 under chapter 3.

4.2.5 Morphology of Fibroblasts on Scaffolds

The cell morphology of in vitro-cultured fibroblasts on PLACL, PLACL-SF, PLACL-SF-AV and PLACL/Collagen nanofibrous scaffolds were analyzed for sixth and ninth day of culture by processing them for FESEM studies as described in section 3.2.5 under chapter 3.

4.2.6 Expression of CMFDA Dye

Fluorescent dye expression was observed in sixth day of fibroblasts cultured on PLACL, PLACL-SF, PLACL-SF-AV and PLACL/Collagen nanofibrous scaffolds using CMFDA dye as described in section 3.2.6 under chapter 3.

4.2.7 Expression of Collagen

Sirius Red staining method for analyzing the presence of collagen on PLACL, PLACL-SF, PLACL-SF-AV and PLACL/Collagen nanofibrous scaffolds secreted by human dermal fibroblast were analyzed as described in section 3.2.7 under chapter 3.

4.2.8 Expression of F-actin Protein

Fibroblast cells stained for F-actin on the sixth day of cell culture on PLACL, PLACL-SF, PLACL-SF-AV and PLACL/Collagen nanofibrous scaffolds were performed as described in section 3.2.8 under chapter 3.
4.2.9 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.

4.3 RESULTS AND DISCUSSION

4.3.1 Characterization of Nanofibrous Scaffolds

The morphology of nanofibrous scaffolds were analyzed by FESEM. Figure 4.1(a-d) shows the FESEM micrographs of PLACL, PLACL-SF, PLACL-SF-AV and PLACL/Collagen nanofibrous scaffolds. The average fiber diameter of PLACL, PLACL-SF, PLACL-SF-AV and PLACL/Collagen nanofibers were in the range of 515±43, 460±32 nm 212±27 and 233±54 nm respectively and results were tabulated in Table 4.1. Among all nanofibrous scaffolds PLACL-SF-AV nanofibers showed smallest diameter range which is due to the addition of AV which significantly decreased the fiber diameter. Previous studies showed that addition of Aloe vera to polymeric solution increases the viscosity and surface tension of the solution resulting in decreased diameter of the resulting fibers (Ibrahim & Arda 2011). Fibroblasts adhered to nanofibers with smaller diameters in comparison to fibers with larger diameters (Tian et al 2008). The FESEM morphology of PLACL-SF-AV is finer when compared to other scaffolds and hence will be more suitable for fibroblast adhesion and proliferation.
The water contact angle parameter was analysed for all the nanofibrous scaffolds and the values are tabulated in Table 4.1. It was found that PLACL nanofibers were hydrophobic having a contact angle of 112º. The contact angles obtained for PLACL-SF, PLACL-SF-AV and PLACL/Collagen were 90º, 52º and 63º respectively. It may be assumed that the observed variations in the contact angle of different nanofibrous scaffolds probably occurred due to structural arrangement of the blend components. The hydrophilic nature of the scaffolds is very important since it is important for cell adhesion and growth (Chen et al 2002). The PLACL-SF-AV scaffold
exhibited better hydrophilic property compared to other scaffolds which may contributed by the wettability and polar nature of AV.

**Table 4.1** Characterization of PLACL, PLACL-SF, PLACL-SF-AV and PLACL/Collagen 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>PLACL</td>
<td>515± 43</td>
<td>112º</td>
<td>1.15</td>
<td>130</td>
<td>25.83</td>
</tr>
<tr>
<td>PLACL-SF</td>
<td>460± 32</td>
<td>90º</td>
<td>1.97</td>
<td>91</td>
<td>12.76</td>
</tr>
<tr>
<td>PLACL-SF-AV</td>
<td>212± 27</td>
<td>52º</td>
<td>0.99</td>
<td>116</td>
<td>12.70</td>
</tr>
<tr>
<td>PLACL/Collagen</td>
<td>233± 54</td>
<td>63º</td>
<td>1.55</td>
<td>55</td>
<td>28.22</td>
</tr>
</tbody>
</table>

Figure 4.2 (a-d) shows the nonlinear stress strain graph of PLACL, PLACL-SF, PLACL-SF-AV and PLACL/Collagen. The tensile stress, strain and elastic modulus values of PLACL, PLACL-SF, PLACL-SF-AV and PLACL/Collagen are given in Table 4.1. Tensile stress for PLACL, PLACL-SF, PLACL-SF-AV and PLACL/Collagen were 1.15 MPa, 1.97 MPa, 0.99 MPa, and 1.55 MPa and has strain of 130 %, 91 %, 116 %, and 55 % respectively. The young’s modulus value for PLACL, PLACL-SF, PLACL-SF-AV and PLACL/Collagen were 25.83 MPa, 12.76 MPa, 12.70 MPa and 28.22 MPa. The data shows incorporation of SF and AV increased the tensile stress and tensile strain values for PLACL-SF-AV nanofibrous scaffolds. The results suggest that blending PLACL with Aloe vera and silk fibroin gives favorable mechanical properties to the nanofibrous scaffolds suitable for
dermal substitutes. The increase in mechanical properties may be due to the reinforcing effect of the blended components.

Figure 4.2  Tensile Stress- strain curves of a) PLACL, b) PLACL-SF, c) PLACL-SF-AV and d) PLACL/Collagen nanofibrous scaffolds

The FTIR spectrum of PLACL, PLACL-SF, PLACL-SF-AV and PLACL/Collagen scaffolds are shown in Figure 4.3(a-d). The C=O vibration occurs at 1749 cm\(^{-1}\) in PLACL nanofibers (Figure 4.3a) The peaks at 1184 cm\(^{-1}\) is due to O-C vibrations. The FTIR spectrum of SF incorporated PLACL scaffolds is shown in spectrum (Figure 4.3b), the absorption bands at 1652, 1535 and 1238 cm\(^{-1}\) corresponds to amide I, amide II and amide III, respectively. The secondary structure of \textit{B. mori} silk fibroin consists of the major conformations including random coils (silk I) and \(\beta\)-sheet (silk II).
Amide I mode is associated with the $\alpha$-helical conformation represented by bands between 1650 and 1660 cm$^{-1}$, the random coil conformations are represented by bands between 1640 and 1650 cm$^{-1}$, and the $\beta$-sheet conformation represented by bands between 1620 cm$^{-1}$ and 1640 cm$^{-1}$. Peaks at 1540 and 1520 cm$^{-1}$ are a random coil and $\beta$-sheet for amide II, respectively, and peaks at 1230 cm$^{-1}$ and 1270 cm$^{-1}$ are a random coil and $\beta$-sheet for amide III, respectively.

![FTIR spectra](image)

**Figure 4.3** The FTIR spectra of a) PLACL, b) PLACL-SF, c) PLACL-SF-AV and d) PLACL/Collagen nanofibrous scaffolds

The spectrum (Figure 4.3c) of PLACL-SF-AV shows the features of spectrum (Figure 4.3b) along with characteristic peaks of *Aloe vera* specifically, the peaks at 1736 cm$^{-1}$ and 1248 cm$^{-1}$ indicates the presence of O-acetyl esters. Furthermore, the absorption band of carboxyl groups at
around 1646 cm\(^{-1}\) (probably an asymmetrical COO stretching vibration or O–H deformation vibration of H\(_2\)O) was found. A further peak at 1032 cm\(^{-1}\) might be due to the glucan units. The pyranoside ring absorption band at 879 cm\(^{-1}\) (C–H ring vibration) and mannose absorption peak at 811 cm\(^{-1}\) were also detected which confirms the successful incorporation of AV in the scaffolds. The FTIR spectrum of PLACL/Collagen scaffold is shown in spectrum (Figure 4.3d) showing the characteristic peaks of collagen along with PLACL. N–H stretching around 3000 cm\(^{-1}\) for amide A, C–H stretching at 3068 cm\(^{-1}\) for amide B, C=O stretching at 1600–1700 cm\(^{-1}\) for amide I, N–H deformation at 1500–1550 cm\(^{-1}\) for amide II and N–H deformation at 1200–1300 cm\(^{-1}\) for amide III band. The amide I, II, and III band regions of the spectrum are directly related to polypeptide conformation confirming the presence of collagen. The spectral result shows the characteristics peaks of silk fibroin, aloe and collagen which confirm the successful incorporation of these agents in to the polymeric backbone.

### 4.3.2 Cell scaffold Interaction and Proliferation

Cell attachment is highly dependent on the nature and surface properties of the scaffolds. The proliferation of fibroblasts on PLACL, PLACL-SF, PLACL-SF-AV and PLACL/Collagen were compared for three different time periods 3, 6 and 9 (Figure 4.4) using MTS assay.

PLACL-SF-AV nanofibrous scaffold shows favorable cells proliferation compared PLACL (control) scaffold which almost increased linearly by \((p \leq 0.01)\) 34.68 % on day 3, \((p \leq 0.01)\) 19.13 % on day 6, and \((p \leq 0.001)\) 97.86 % on day 9. Compared to the PLACL-SF scaffolds, the SF/AV blended scaffold showed significantly enhanced proliferation about \((p \leq 0.001)\) 41.12 % and 23.01 % on day 3 and 6. Similarly with
PLACL/Collagen, PLACL-SF-AV scaffold showed significant increase of about \((p \leq 0.01) 16.23\% \) and \((p \leq 0.001) 26.88\%\) on day 6 and 9. Mannose 6-phosphate and acemannan of *Aloe vera* are the important components responsible for increased proliferation of human fibroblast cells on PLACL-SF-AV scaffold which mediate cell signalling pathway for proliferation of fibroblasts (Stashak & Theoret 2008, Davis et al 1994).

![Figure 4.4](image)

**Figure 4.4** MTS assay for human fibroblast proliferation on a) TCP, b) PLACL, c) PLACL-SF, d) PLACL-SF-AV and e) PLACL/Collagen nanofibrous scaffolds on day 3, 6 and 9. * indicates significant difference of \(p \leq 0.01\); ** indicates significant difference of \(p \leq 0.001\)
Figure 4.5 FESEM micrographs of fibroblast interaction with nanofibrous scaffolds after 6 days of culture on a) TCP, b) PLACL, c) PLACL-SF, d) PLACL-SF-AV and e) PLACL/Collagen nanofibrous scaffolds at 500 x magnifications

The FESEM images (Figure 4.5 (a-e) and Figure 4.6 (a-e)) of cells grown on PLACL, PLACL-SF, PLACL-SF-AV and PLACL/Collagen scaffolds supported the MTS data. PLACL-SF-AV scaffolds showed the normal spindle shaped morphology and higher cell growth of human fibroblast cells on day 3, 6 and 9 when compared to other scaffolds and favored the cells migration along the fibers orientation.
Figure 4.6 FESEM micrographs of fibroblast interaction with nanofibrous scaffolds after 9 days of culture on a) TCP, b) PLACL, c) PLACL-SF, d) PLACL-SF-AV and e) PLACL/Collagen nanofibrous scaffolds at 500 x magnifications

Mammalian cell surface is negatively charged, positively charged arginine residue on silk fibroin sequence, may help in cell attachment and Glucomannan, a mannose-rich polysaccharide of Aloe vera through binding with basic fibroblast growth factor (bFGF) receptor or altering signalling pathways for bFGF (factor for wound healing) brings enhanced cell proliferation (Talukdar et al 2011, Chithra et al 1998) Therefore, the enhancement of skin fibroblast proliferation found to be due to combined properties of silk fibroin and Aloe vera which aids in cell attachment and proliferation respectively. The hydrophilic nature and favorable mechanical
property of PLACL-SF-AV scaffolds is another reason for better adhesion, migration and proliferation of fibroblasts for skin tissue regeneration.

4.3.3 Expression of CMFDA, Collagen and F-actin

CMFDA stained fibroblast was observed on the sixth day of fibroblast culture using a Leica fluorescence microscope. The PLACL-SF-AV scaffolds showed comparatively increased fibroblasts density and morphology than the other nanofibrous scaffolds. Figure 4.7 (a-e) shows the CMFDA stained fibroblast (green) cells on different scaffolds.

![CMFDA stained fibroblast cells on different scaffolds](image)

Figure 4.7 The detection of CMFDA-labeled fibroblasts on day 6 of cell culture a) TCP, b) PLACL, c) PLACL-SF, d) PLACL-SF-AV and e) PLACL/Collagen nanofibrous scaffolds at 10x magnifications

Collagen is one of the body’s key natural resources and a component of skin tissue that can benefit all stages of the wound healing
process. When collagen is made available to the wound bed, closure can occur. An ideal skin tissue regeneration scaffold should induce fibroblast for collagen secretion (Venugopal et al 2006).

Figure 4.8 Optical microscope images showing the secretion of Collagen by fibroblast cells on day 6 using picro-sirius red staining on a) TCP, b) PLACL, c) PLACL-SF, d) PLACL-SF-AV and e) PLACL/Collagen nanofibrous scaffolds at 10x magnifications

Figure 4.8 (a-e) and Figure 4.9 (a-e) shows the secretion of collagen on fibroblasts grown on TCP, PLACL, PLACL-SF, PLACL-SF-AV, PLACL/Collagen nanofibrous scaffolds on day 6 and 9 respectively. Collagen stained with Picro-sirius red confirmed the secretion of the collagen by the cells in culture. The results showed increased secretion of collagen on PLACL-SF-AV compared to all other scaffolds. Previous studies shows AV derived Glucomannan, a mannose-rich polysaccharide, and gibberellin, a
growth hormone, interacts with growth factor receptors on the fibroblast, thereby stimulating its activity and proliferation, which in turn significantly increases collagen synthesis after topical and oral application (Chithra et al 1998). The result proves that the biocomposite nanofibrous PLACL-SF-AV scaffold have potential role for wound healing through skin tissue regeneration.

![Figure 4.9 Optical microscope images showing the secretion of Collagen by fibroblast cells on day 9 using picro-sirius red staining on a) TCP, b) PLACL, c) PLACL-SF, d) PLACL-SF-AV and e) PLACL/Collagen nanofibrous scaffolds at 10x magnifications](image-url)
Figure 4.10  F-actin expression of fibroblast cells on a) TCP, b) PLACL, c) PLACL-SF, d) PLACL-SF-AV and e) PLACL/Collagen nanofibrous scaffolds at 60x magnification. The cell cytoskeleton were labelled with TRITC conjugated phalloidin (red) and nucleus with DAPI (blue)

Staining for cytoskeleton protein, F-actin of human primary fibroblasts cultured on the TCP and scaffolds were shown in Figure 4.10 (a-e). The cell cytoskeleton were labelled with TRITC conjugated phalloidin and nucleus with DAPI and visualized by confocal microscopy. DAPI binds mainly to double-stranded DNA in the cell nucleus, emitting a blue fluorescence when exposed to ultraviolet light this enabled the identification of individual fibroblasts present within the scaffold visualized using confocal microscopy, whereby cell numbers could be quantified based on the confocal images obtained. A second stain, phalloidin, was included in order to differentiate the filamentous actin (F-actin) within fibroblasts. With its close affinity to the groove between actin subunits in F-actin, phalloidin
serves as an effective marker of polymerised F-actin when the conjugated Alexa-488 probe on phalloidin gives off a red fluorescence, upon excitation changes in cell morphology and shape are influenced largely by reorganization of actin filaments (Venugopal & Ramakrishna 2005). Increased levels of filamentous actin expression correlated with higher contractile activity in fibroblasts (Miki et al 2000). Fibroblasts grown in PLACL-SF-AV scaffold with more elongated morphology showed different filamentous actin levels than the other scaffolds. This suggests that cells with elongated morphology on PLACL-SF-AV scaffold may have higher contractile behavior due to higher levels of filamentous actin that may enhance faster attachment, proliferation and guided growth on the PLACL-SF-AV nanofibers for skin tissue engineering.

4.4 CONCLUSIONS

Electrospinning of multiple natural polymer blends can yield a mixture of natural nanofibers that closely mimic the native ECM. A large percentage of native tissues contains both protein and polysaccharides fibers that are frequently subjected to tensile and elastic loading, respectively. Electrospun fibrous scaffolds composed of silk fibroin and Aloe vera have been fabricated to replicate the native ECM of human skin. PLACL-SF-AV scaffold supports fibroblast proliferation and produces distinct phenotypic differences in the fibroblasts. The fibroblasts adopted an elongated morphology when grown in PLACL-SF-AV that may simulate the large disruptions in ECM present at a wound site. Human fibroblast cells could adhere and proliferate well on these composite matrixes, which could be probably correlated to the different components present with Aloe vera promoting cell proliferation and silk fibroin providing favorable stable environment for cell adhesion and migration which ultimately promoted the regenerative process of the human fibroblast cells suitable for dermal
application. This herbal biodegradable scaffolds are promising candidates for the treatment of different skin disorders or damages with less immunogenic responses. The PLACL-SF-AV scaffolds in comparison with the previous collagen/gelatin based scaffolds will serve as better tissue engineering scaffolds in the longer run because of the relatively low cost and higher mechanical stability which are typically required for dermal applications.