CHAPTER 2

CROSSLINKING OF TYPE I COLLAGEN ON POLYCAPROLACTONE NANOFIBERS FOR TISSUE ENGINEERING

2.1 INTRODUCTION

Tissue engineering is an interdisciplinary technology that combines knowledge from material engineering, cellular biology, and genetic engineering to produce innovative three-dimensional composites having structural/functional properties that can be used either to replace or correct poorly functioning components in humans and animals, such as skin, cartilage for joints, heart valves, bone, etc. or to introduce better functional components into these living systems. A functional scaffold for tissue engineering must support and define the three-dimensional organization of the tissue-engineered space and, at the same time, maintain the normal differentiated state of the cells within the cellular compartment. Ideally, a functional scaffold should be able to mimic the structure and biological function of the native extracellular matrix (ECM) proteins, to provide mechanical support and regulate cellular activities (Ratner et al 2004, Mitchell & Davis 2011).

The continuous structured nature of fibers make them a suitable agent for fabrication into biomaterial scaffolds for tissue engineering, since fibrous structures can morphologically resemble ECM components in tissues. Fibrous scaffolds are attractive for tissue engineering because of their inherent advantages of high surface area for cell attachment, controlled porous
architecture, and a 3-D microenvironment for cell-cell contact. Conventional fibers produced by mechanical spinning generally measure tens of microns in diameter. Such fibers have relatively low specific surface area, and their diameters are far larger than the diameters normally encountered in nature. Smaller, submicron-diameter polymeric fibers, or nanofibers, may provide stronger topographic cues by mimicking the filamentary ECM. Nanofibers composed of natural or biodegradable polymers can also be tailored to possess the tissue-matching mechanical compliance (Chew et al. 2006). With the advancements in scaffold designing, electrospinning has evolved as a major technology in the production of nanofibrous scaffolds in the past several years with materials of different compositions for applications of varying end uses in tissue engineering (Li et al. 2002, Yoshimoto et al. 2003, Chen et al. 2011b).

Electrospinning in tissue engineering has come to prominence in the previous two decades. Electrospinning is a relatively simple and inexpensive scaffold fabrication process in the form of non-woven membranes. Because of the high surface area to volume or mass ratio and the vast possibilities for surface functionalization, electrospun fibers have become the most studied form of tissue-engineered scaffolds (Li et al. 2002).

2.1.1 Electrospinning

Electrospinning, also known as electrostatic fiber processing, technique has been originally developed for generating ultrathin polymer fibers using electric forces. The electrospinning technology is well suited to process natural biomaterials and synthetic biocompatible or bio-absorbable nanofibers for biomedical applications. Electrospinning, patented by Formhals in 1934, enables polymeric fibers with diameters in the range of a few nanometers to several microns to be fabricated, depending on the type of polymer and the processing conditions. In electrospinning, a high voltage is
used to create an electrically charged jet of polymer solution or melt out of the pipette. High voltage potential is applied to the tip of the needle, thereby inducing free charges into the polymer solution. These charged ions move in response to the applied electric field towards the electrode of opposite polarity, thereby transferring tensile forces to the polymer solution. At the tip of the needle, the polymer drop takes a cone like projection in the presence of an electric field called as “Taylor cone”. And, when the applied potential reaches a critical value required to overcome the surface tension of the liquid, a jet of liquid is ejected from the cone tip.

After the initiation from the taylor cone, the discharged polymer solution jet undergoes an instability and elongation process, which allows the jet to become very long, thin and is directed towards the oppositely charged collector. As the jet travels through the atmosphere, the solvent evaporates, leaving behind a dry fiber on the collecting device (Li et al 2002, Li & Xia 2004). Various synthetic and natural polymeric fibers with diameters ranging from nanoscale to microscale have been successfully produced by electrospinning. The morphology of the fibers depends on the process parameters, including solution concentration, applied electric field strength, and the feeding rate of the precursor solution (Doshi & Reneker 1995, Boudriot et al 2006, Pham et al 2006).

Electrospun nanofibrous scaffolds possess an extremely high surface-to-volume ratio, tunable porosity, and malleability to conform over a wide variety of sizes and shapes. In addition, the scaffold composition can be controlled to achieve desired properties and functionality (Ma et al 2005b). The topographical and architectural arrangement of the nanofibrous scaffolds paves a way for good tissue engineering material, provided the polymer used is biodegradable, biocompatible and non-toxic to the cells (Cao et al 2010).
2.1.2 Surface Modification of Electrospun Nanofibers

Natural polymers that have been electrospun are proteins and polysaccharides. There are several challenges to be encountered in the use of naturally derived polymers, like batch-to-batch (or source-to-source) variation in materials isolated from tissues, restricted flexibility in the range of accessible material properties, mechanical strength, and therefore artificial polymers were synthesized as alternative materials for biomedical applications. However, the optimal biomedical application of the electrospun nanofibers would be achieved by using synthetic polymers in combination with natural polymers to mimic a matrix compatible for tissue engineering and regeneration (Liao et al 2006).

The “hybrid” combinations of natural and synthetic materials confer “biological functionality” to the synthetic biomaterial. Although the combined use of different types of polymers can significantly improve the physical and biological properties of nanofibrous scaffolds, further modification of their surface with specific bio-functional groups is often needed in order to improve their in-vivo usage (Li et al 2006). Specifically, surface modification of electrospun nanofibers with proteins or peptides is essential to develop functional nanofibers with more desirable biological features for tissue engineering applications (Zhang et al 2007, Rim et al 2013).

Among the different classes of synthetic polymers that could be biologically modified, polyesters are especially interesting because their surfaces may contain reactive groups de novo, or they may be readily derivatized with reactive groups that can be used to covalently link biomolecules. Another advantage of polyorthoesters as supports for biomolecules is that the polymers may be fabricated in many forms, including
films, membranes, tubes, fibers, fabrics, particles, capsules, and porous structures (Ratner et al 2004).

The scaffolds from synthetic polymer (like polyester) are versatile in achieving the desirable structural and mechanical properties but lack cytocompatibility and do not mimic the natural architecture of the ECM, which establishes the fact that synthetic polymers are less efficient as constructing material in tissue engineering (Pham et al 2006).

Surface modification of polymers (synthetic) has long been recognized as a potential tool for achieving or enhancing the biocompatibility and surface properties of the material. Functional groups for crosslinking of biomolecule may already be present or can be added to the surface by various chemical treatments depending on the molecular configuration of the specific polymer used. Many different biologically functional molecules can be chemically or physically (etching, mechanically roughening, over coating, grafting, and thin film deposition, etc.,) immobilized on polymeric supports. For chemical (covalent) bonding to an inert solid polymer surface, the surface must first be chemically modified to provide reactive groups (e.g., –OH, –NH₂, –COOH, –SH, or –CH=CH₂) for the subsequent immobilization using crosslinking agent. Carboxyl and amino groups are the most commonly used functional groups in surface modification of synthetic polymers. If the polymer support does not contain such groups, then it is necessary to modify it in order to permit covalent immobilization of biomolecules to the surface (Ratner et al 2004).

Polycaprolactone (PCL), an ideal polyester scaffolding material owing to its biodegradability and biocompatibility, is a semicrystalline polymer with a melting temperature \((T_m)\) of 60°C and a glass transition temperature \((T_g)\) of -60°C. PCL, approved by the U.S. Food and Drug Administration (FDA), has good mechanical properties when bi-axially stretched (Mattanavee et al. 2009). Extensive studies have been conducted on the polycaprolactone (PCL), in tissue engineering applications viz., cartilage (Woodfield et al. 2004, Casper et al. 2010), bone (Fujihara et al. 2005, Mavis et al. 2009), skin (Venugopal & Ramakrishna 2005), and drug delivery implants for controlled release of drugs for longer periods (Kim et al. 2010, Yoon & Kim 2011). It is well known that synthetic polymers can be designed to confer tunable chemical and mechanical properties, but they usually lack bio-functionality. This often leads to poor cell affinity and limited cell proliferation, and it hinders active control of cellular responses (Rim et al. 2013). Notwithstanding its hydrophobicity often leads to unfavorable cell adhesion and growth. Therefore, the cyto-compatibility of PCL should be further improved.

The co-spinning of PCL with various natural polymers/protein to mimic ECM drew wide attention (Kwon & Matsuda 2005, Zhang et al. 2005, Li et al. 2006), but the complex and uncontrollable methods to maintain size and porosity remains an impediment. Consequently, the surface modification of electrospun PCL nanofibers with natural polymer/protein becomes a viable option to improve the hydrophilicity and compatibility for better proliferation and subsequent infiltration of cells. Recent reports concludes that hydrophilic and protein containing surfaces are known to promote cellular growth (Mattanavee et al. 2009).

Collagen is the most abundant protein in animals used for tissue engineering. Because of its abundance, ubiquity, biocompatibility and the
presence of cell binding sequences such as Arg-Gly-Asp (RGD) and Gly-Phe-Hyp-Gly-Glu-Arg (GFOGER), collagen has become the most preferred biopolymer for surface functionalization compared to other natural polymers. (Chiu et al 2007, Khew & Tong 2007, Carson & Barker 2009, Yoo et al 2009).

Among the various surface modification techniques, aminolysis (wet chemical) is one of the simplest ways to conduct surface modification of PCL for better interaction of cells. There are abundant ester groups (-COO-) in PCL molecules. These ester groups can be hydrolyzed to carboxylic acid under alkaline conditions. In addition, it is possible to introduce amino groups onto the polyester surface by a reaction with diamine like ethylenediamine (EDA) and hexanediamine (HDA); where one amino group reacts with the -COO- group to form a covalent bond (-CONH-) and the other amino group remains free, which can be further crosslinked with biopolymers (Zhu et al 2002a, Zhu et al 2002b, Zhu et al 2004c). The modified functionality of the surface was found to improve cell interaction and proliferation of various cells in the matrix.

In this chapter we present the crosslinking of collagen on to the PCL nanofibers to enhance the biological characteristics of the PCL nanofibers.

The present contribution focuses on improving the hydrophilicity, and hence the cyto-compatibility, of the surface of the electrospun PCL fibrous scaffolds. The prepared scaffold anticipated to mimic ECM was characterized for its effect on cell adhesion using fibroblast cells.
2.2 MATERIALS

Materials used in the fabrication of the fibrous scaffolds were polycaprolactone (PCL; Mn- 80,000 gmol\(^{-1}\) from Aldrich), dichloromethane (DCM; Carlo Erba, Rodano, Italy), and \(N,N'\)-dimethylformamide (DMF; Labscan Asia, Bangkok, Thailand). Materials used in the modification of the surface of the PCL fibrous scaffolds were Type I collagen purified from bovine achilles tendon (Aishwarya S et al 2007), 1,6-hexamethylene diamine (HMD) (Fluka, Buchs, Switzerland), and \(N,N'\)-disuccinimidyl carbonate (Novabiochem, Merck, Haar, Germany). All other chemicals were of analytical reagent grade and used without further purification.

2.3 METHODS

2.3.1 Preparation of PCL Nanofibers

Nano-fibrous PCL scaffolds were prepared using electrospinning technique in the conditions described below. PCL were dissolved in mixed solvent at 7:3 ratio of chloroform: dimethylformamide and stirred to obtain a homogeneous solution of 10\%, 12.5\% and 15\% w/v. For the process of electrospinning, the polymeric solution was placed in a burette with tip diameter of 1.2 mm. The positive electrode of the high-voltage power supply was clamped directly to a blunt needle. The solution was electrospun at the rate of 3 mL h\(^{-1}\) onto a grounded stainless steel plate covered with aluminium foil kept at a distance of 10 cm from the burette tip. In order to make membranes of even-thickness, the collector was moved to and fro in an ordered fashion for all the three concentrations. The electrical strength applied for electrospinning PCL was 30 kV cm\(^{-1}\). All electrospun fibrous membranes were vacuum dried and stored in desiccators.
2.3.2 **Fiber Morphology and Size Analysis Using SEM**

The morphology of PCL nanofibers were investigated using scanning electron microscope (SEM, JEOL, Tokyo, Japan) at an accelerating voltage of 15 kV. Samples were mounted onto stubs and coated with gold using a sputter coater (JEOL, Tokyo, Japan). The diameters of resulting nanofibers were analyzed using software Image J.

2.3.3 **Porosity Analysis**

The apparent density of the electrospun scaffold was accurately measured using density bottle method. An average of three measurements was taken for each sample. The porosity of electrospinning nanofibrous scaffold was calculated by using the following equation (2.1) and (2.2) (Chong et al 2007, Meng et al 2010).

\[
\text{Porosity of scaffold} (\%) = 1 - \left( \frac{\text{Apparent density of scaffold (g cm}^{-3})}{\text{Bulk density of Polycaprolactone (g cm}^{-3})} \right) \times 100 
\]  \hspace{1cm} (2.1)

\[
\text{Apparent density of scaffold (g cm}^{-3}) = \frac{\text{Mass of scaffold (g)}}{\text{Scaffold thickness (cm) \times scaffold area (cm}^2)} \]  \hspace{1cm} (2.2)

2.3.4 **Surface Modification of PCL Nanofibers**

Surface modification of PCL nanofibers was carried out in two stages viz., (a) aminolysis and (b) collagen grafting.

(a) Aminolysis of PCL nanofibers: PCL nanofibers were cut into pieces and immersed in ethanol/water mixture (prepared in the ratio of 1:1) for 2-3 h to clean oil, dirt and then washed with a
large amount of deionised water. Subsequently, the washed nanofibrous membrane was immersed in 50 mL of 1,6-hexanediame solution (10% w/v prepared in 2-propanol) for 12 h at 37°C. After treatment, the membrane was rinsed with deionised water for 24 h at room temperature to remove free 1,6-hexanediame and dried in a vacuum at 30°C for 24 h to constant weight (Zhu et al 2002b, Zhu et al 2004b, Zhu et al 2004c). Energy Dispersive X-ray analysis (EDAX) was conducted to investigate the existence and distribution of nitrogen in the chemically modified PCL nanofibers as a proof to confirm the introduction of amino groups by aminolysis and the surface analysis of PCL and aminolysed PCL nanofibers (APN) were carried out. The carbon, hydrogen, nitrogen ratios were determined in different regions of the samples using an energy dispersive X-ray analysis (EDAX) detector connected to a scanning electron microscope (SEM) (JEOL, Tokyo, Japan).

(b) Collagen Immobilisation: The APN were immersed in 1% w/v glutaraldehyde (GA) solution for 3 h at room temperature, followed by rinsing 5 - 6 time with 50 mL of deionised water to remove free GA. The GA treated PCL nanofibers were further incubated in collagen solution (pH 3.4, 5mg mL⁻¹) for 24 h at 2 - 4°C. The collagen treated nanofibers were soaked in 0.5M acetic acid solution for 2 h to remove the free collagen molecules, rinsed with deionised water and dried at room temperature (Zhu et al 2004b).
2.3.5 Attenuated Total Reflectance - Fourier Transform Infrared (ATR-FTIR) Spectroscopy

To characterize the surface properties for APN and collagen crosslinked PCL nanofibers (CCPN), Attenuated total reflectance - Fourier transform infrared (ATR-FTIR, Bruker Tensor, Bruker, USA) spectroscopic investigation was carried out.

2.3.6 Water Contact Angle Measurement

Water contact angles of the PCL Nanofibers, APN and CCPN were measured at room temperature and 60% Relative humidity (RH), using sessile drop method on a Goniometer (VCA Optima Pvt. Ltd). A distilled water drop is put on the surface of nanofibrous membranes at five different sites and the measured angles were averaged.

2.3.7 Tensile Strength Measurements

Tensile strength of PCL nanofibers and surface modified PCL nanofibers were tested using INSTRON instruments series II Automated materials testing system. The PCL nanofibers were cut into dumbbell shaped strips with 40 mm length and 5 mm inner width. The thicknesses of the samples were measured using the electronic digital micrometer screw gauge. The sample strips were held by pneumatic grips and tensile force was applied at an extension rate of 2 mm/min. The tests were conducted at 20 ± 2°C in 65 ± 5% RH. Triplicates were tested for each type of electrospun nanofibrous membranes.

2.3.8 NIH 3T3 Fibroblast Culture Studies

Fibroblasts were cultured in dulbecco’s modified eagle’s medium (DMEM/F12) (1:1) containing 10% fetal bovine serum (FBS) in 75 cm²
flasks. The culture was maintained at 37ºC in a humidified CO₂ incubator for 7 days and NIH 3T3 fibroblasts were harvested by trypsin-ethylene diamine tetra acetic acid (Trypsin–EDTA) treatment and utilised for in vitro studies.

2.3.8.1 Processing of NIH 3T3 fibroblasts seeded scaffolds for SEM

NIH 3T3 Fibroblasts were seeded (1×10⁴ cells cm⁻²) on PCL nanofibers, aminolysed PCL nanofibers, and collagen crosslinked PCL nanofibers on 24 well plates. After 48 h, the scaffolds were washed with PBS to remove non-adherent NIH 3T3 cells and then fixed in 2.5% glutaraldehyde for 2h at room temperature, dehydrated through a series of graded alcohol solutions and finally dried overnight (Zhang et al 2005). Dried cellular constructs were sputter coated with gold (JEOL JFC-1600 Auto fine Coater, Japan) and observed under the SEM (JEOL JSM-5800LV, Japan) at an accelerating voltage of 10 kV.

2.3.8.2 MTT proliferation assay

NIH 3T3 fibroblasts were seeded (1×10⁴ cells cm⁻²) on PCL nanofibers, aminolysed PCL nanofibers, and PCL-collagen on 96 well plates. Cell proliferation was monitored at 24 h and 48 h by MTT assay (Truong et al 2010). In order to monitor cell adhesion and proliferation rate on different substrates, the number of cells was determined using the colorimetric MTT assay. Metabolically active cells react with a tetrazolium salt in the MTT reagent to produce purple coloured crystals that are then released from the cells by lysis using dimethylsulphoxide (DMSO) and this was observed at 560 nm. The cellular constructs were rinsed with PBS followed by incubation with 20% MTT reagent in serum free medium for 3 hand aliquots were pipetted out and spectrophotometrically read at 560 nm.
2.3.9 Statistical Analysis

The quantitative assays were analysed statistically using one way ANOVA. The data were presented as mean ± standard deviation (SD). P-value < 0.05 were considered statistically significant. All statistical procedures were performed using a software package Graph Pad Prism 5.

2.4 RESULTS AND DISCUSSION

Currently PCL, a synthetic biodegradable polymer, is widely used in controlled drug delivery systems and tissue engineering. The PCL has advantages over other biodegradable polymers like slow biodegradation rate and being cost effective. The relatively low biocompatibility of this polymer due to its hydrophobic properties could be altered with the biomolecule crosslinking on the surface and it can be widely used for tissue engineering applications.

The electrospinning process can be manipulated by a number of variables as mentioned earlier in section 2.1.1. PCL with a molecular weight of 80,000 g mol\(^{-1}\) has been selected for electrospinning, because it was observed that the increasing molecular weight may result in reduction in number of bead and droplet formation (Pham et al 2006). In general, it was found that lower flow rates yielded fibers with smaller diameters. So the rate of flow was kept at 3 mL h\(^{-1}\). Varying the distance between the tip and the collector has been examined to control the fiber diameters and morphology. It has been found that a minimum distance is required to allow the fibers to dry before reaching the collector (Geng et al 2005, Ki et al 2005). At distances that are either too close or too far, beading has been observed. Here for PCL, a distance of 10 cm has been maintained between the tip and collector. In this study, we used Chloroform/DMF as a solvent for the electrospinning process and fabrication of the fibrous scaffolds. Chloroform/ DMF mixture is an ideal
organic solvent, in that it allows full extension of the polymer and it evaporates completely after the fiber formation process without leaving any residue on the formed fibers.

2.4.1 Optimization of PCL Concentration

The PCL concentration of 10, 12.5 and 15% w/v solution has been tried to check its effect in morphology and diameter. The photographic image of the PCL nanofibers produced by spinning 10% of PCL is shown in Figure 2.1.

![The photographic image of electrospun PCL nanofibers 10% w/v](image)

All electrospun PCL nanofibrous scaffolds appeared relatively uniform and smooth except the 15% PCL concentration when observed under SEM is shown in Figures 2.2 – 2.4. The diameter of the PCL nanofiber was estimated for all the three concentration using image analysis software (Image J, National Institutes of Health, Bethesda, MD) and the results are given in Table 2.1. At 10% PCL concentration (Figure 2.2), there is no evidence of bead formation and the pore distribution displayed the good interconnection between fibers compared to 12.5 and 15% of PCL concentration. The average
fiber diameter of 10, 12.5 and 15% (w/v) of PCL (Figure 2.3) concentration were found to be 261, 312 and 402 nm respectively. The increase in concentration of the PCL from 10% to 12.5% did not show any significant (P>0.05) increase in fiber diameter, whereas the 15% PCL concentration showed a significant (P<0.001) increase in diameter compared to that of 10% and 12.5%. The SEM images of nanofibrous mats of 12.5% PCL did not show any bead formation compared to that of 15% in which there was presence of beads at regular intervals in the nanofibers. The 10% and 15% fibers had good intersections, whereas it was poor in 12.5% PCL. The increase in fiber diameter can be attributed to the increase in concentration of the polymer solution increased the viscosity.

Table 2.1  Effect of PCL concentration on the morphology and porosity of PCL nanofibrous scaffold (* indicates Significant difference compared to that of 10% PCL and # indicates significant difference to that of 12.5% PCL, n=25)

<table>
<thead>
<tr>
<th>PCL Concentration (w/v)</th>
<th>Fiber Diameter Mean ± SD (nm)</th>
<th>Morphology</th>
<th>Porosity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10%</td>
<td>261.2 ± 88.7</td>
<td>Fibrous, High intersections, No bead formation</td>
<td>65%</td>
</tr>
<tr>
<td>12.5%</td>
<td>312.9 ± 87.3</td>
<td>Fibrous, Low intersections, No bead formation</td>
<td>59%</td>
</tr>
<tr>
<td>15%</td>
<td>402.8 ± 106.3*#</td>
<td>Thick Fibers, High intersections, Beads at regular intervals</td>
<td>59%</td>
</tr>
</tbody>
</table>

The increasing polymer concentration (15%) yielded uniform fibers but bead at regular distances were found (Figure 2.4). The porosity of the electrospun PCL nanofibrous scaffolds in this study is estimated to be around 59–65%. In spite of variation in concentration of polymer there is not much difference in porosity. It is not easy to create well-defined pore sizes through the electrospinning technique because of the randomly deposited fibers.
Figure 2.2  The SEM morphology of electrospun PCL nanofibers at 10% w/v PCL concentration

Figure 2.3  The SEM morphology of electrospun PCL nanofibers at 12.5% w/v PCL concentration
However, the overall network architecture structure fabricated best mimics the natural ECM. The pores generated within the scaffold structure for cellular in-growth are formed by nanofibers lying loosely upon each other which are different from isotropic pores made by using particles or bubbles when the scaffold is solidified. The presence of good interconnections, more porosity and lesser scale in dimensions (diameter) compared to others helped to choose 10% PCL as optimal scaffold for tissue engineering and has been selected for further modifications.

### 2.4.2 Aminolysis of PCL Nanofibers

Scaffolds of PCL nanofibers were prepared using electrospinning technique. The prepared fibers were aminolysed as per the procedure described by Zhu et al (2004b). The aminolysis of PCL nanofibers were confirmed using EDAX by the detection of nitrogen in the aminolysed PCL
nanofibers. As the PCL polymer does not have nitrogen in its native form, the very presence of nitrogen in the modified sample confirms the successful aminolysis of PCL nanofibers. The EDAX spectrum was conducted to confirm the presence of nitrogen in the aminolysed nanofibers and the results are given in Table 2.2. The EDAX spectrum of the unmodified PCL nanofibers showed only the presence of carbon and oxygen, whereas in case of EDAX of aminolysed PCL nanofibers, nitrogen was detected in addition to carbon and oxygen. This observation confirmed that the nitrogen (of NH$_2$) is introduced to the electrospun PCL nanofibers by aminolysis.

Table 2.2  Elemental analysis of aminolysed PCL nanofibers using Energy dispersive X-ray analysis (EDAX)

<table>
<thead>
<tr>
<th>EDAX</th>
<th>Elements Weight %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Carbon</td>
</tr>
<tr>
<td>Polycaprolactone Nanofibers</td>
<td>69.62</td>
</tr>
<tr>
<td>Aminolysed PCL Nanofibers</td>
<td>57.08</td>
</tr>
</tbody>
</table>

Acc.Voltage: 15.0 kV  Take Off Angle: 35.0 deg.

2.4.3  Characterisation of PCL Nanofibers and Collagen Crosslinking

Surface morphologies of the PCL nanofibers were characterized using SEM. The SEM images of PCL nanofibers, APN and CCPN are shown in Figure 2.5 as A, B and C respectively. The diameter of the fibers in APN and CCPN were found to be 213.88 ± 74.56 nm and 368.67 ± 108.38 nm respectively. APN fiber diameter does not seem to be affected appreciably by aminolysis (P>0.05) and collagen crosslinking caused a significant (P<0.001) increase in fiber diameter which may be due to simple adsorption of collagen in the surface of fibers. The surface of CCPN also showed a non-uniformity
and can able to clearly observe the collagen coating (adsorbed) on the surfaces of APN.

ATR-FTIR spectra (500–4000 cm$^{-1}$) of PCL nanofibers and CCPN are shown in Figure 2.6. In the spectra of PCL nanofibers (Figure 2.6a) and CCPN (Figure 2.6b), there is a distinctive absorptive band at 1722.47 cm$^{-1}$ due to the abundant ester C=O bonds in the structure of the PCL.

![Figure 2.5](image)

**Figure 2.5** The scanning electron microscopic images of A) Native PCL nanofibers, B) Aminolysed PCL nanofibers and C) Collagen crosslinked PCL nanofibers
When collagen is crosslinked onto the surface of the aminolysed PCL nanofibers, three distinct peaks around 1556, 1661 and 3325 cm\(^{-1}\) were observed. The amide A band of collagen (associated with the NH-stretching frequency) is usually found at 3325–3330 cm\(^{-1}\). Peak at 3324.98 cm\(^{-1}\) was observed for collagen crosslinked PCL nanofibers corresponds to NH stretch and the broadening of the peak could be due to the hydrogen bonding associated with the collagen.

![ATR-FTIR spectra of nanofibers](image)

**Figure 2.6** ATR-FTIR spectra of nanofibers a) Native PCL nanofibers, b) Collagen crosslinked PCL nanofibers

The amide I band of collagen is usually observed around 1650–1668 cm\(^{-1}\) corresponding to C=O stretch and amide II band of collagen is centred in the range 1530–1555 cm\(^{-1}\), which corresponds to NH stretch coupled with CN stretch. In our samples of collagen crosslinked PCL nanofiber, Amide I and Amide II appeared at 1661 and 1556 cm\(^{-1}\)
respectively (Yang et al 2002). Hence, it is clear that collagen has been successfully immobilised on to the PCL nanofiber by crosslinking using glutaraldehyde.

The water contact angle of unmodified PCL nanofiber scaffold was measured as $92.81 \pm 0.39$ degree. This clearly shows that PCL is highly hydrophobic in nature. The contact angle extensively reduced after aminolysis due to introduction of NH$_2$ groups and subsequent collagen coating using glutaraldehyde crosslinking (Lin et al 1994). The water contact angle of unmodified nanofibers, aminolysed nanofibers, and collagen cross linked nanofibers are $92.81 \pm 0.39$, $50 \pm 0.25$ and $0$ degrees, respectively. This reflects the enhanced hydrophilicity of the surface due to its modification after aminolysis. The results of post aminolysis proved the role of NH$_2$ groups in increasing the hydrophilicity of the surface (Lin et al 1994, Shabani et al 2009). The water contact angle has been observed to reduce to zero after collagen grafting and similar observation has been reported earlier (Shabani et al 2009). Thus, the very objective of hydrophobicity reduction by the modification of PCL nanofiber scaffold for better biocompatibility and infiltration of cells for effective tissue engineering has been achieved.

The mechanical strength of all the samples were analysed and the results are shown in the Table 2.3. From the results, it is observed that the tensile strength of PCL nanofibers is not significantly ($P>0.05$) higher than the aminolysed PCL nanofibers. The decrease in tensile strength of modified PCL nanofibers may be due to polymer erosion leading to weight loss, which is an unavoidable consequence during aminolysis process using hexanedianmine.
Table 2.3  Effect of surface modification on tensile strength of PCL nanofibers (# - indicates significant difference compared to that of aminolysed PCL nanofibers, n=3)

<table>
<thead>
<tr>
<th>Nanofibers</th>
<th>Tensile Strength</th>
<th>Mean ± SD (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polycaprolactone Nanofibers</td>
<td></td>
<td>3.51 ± 0.989</td>
</tr>
<tr>
<td>Aminolysed Polycaprolactone Nanofibers</td>
<td></td>
<td>1.95 ± 0.136</td>
</tr>
<tr>
<td>Collagen crosslinked Polycaprolactone Nanofibers</td>
<td></td>
<td>4.06 ± 0.523*</td>
</tr>
</tbody>
</table>

Similar observations have been noted by Hong et al (2005). In spite of decreased tensile strength of PCL nanofibers due to aminolysis, the collagen crosslinking has facilitated the PCL nanofibers to regain its lost tensile strength and it is significantly higher compared to that of aminolysed PCL nanofibers. An efficient scaffold should have good mechanical properties when employed as bone, cartilage, cardiac constructs, etc., for effective tissue engineering applications. Irrespective of the impact of aminolysis on the mechanical properties of PCL nanofibers, collagen immobilisation has shown its advantage to regain the mechanical properties.

2.4.4  Fibroblasts Attachment to Modified PCL Nanofibers

In our present study, the NIH 3T3 fibroblast were seeded in the three different scaffolds viz., PCL nanofibers, aminolysed PCL nanofibers, and collagen coated PCL nanofibers for 48 h. All samples were both quantitatively (MTT) and qualitatively (SEM) analysed for studying the cellular behaviour of the prepared scaffolds.

The qualitative studies on cellular proliferation and infiltration were carried out by SEM analysis. The SEM images (Figure 2.7) show that NIH
Figure 2.7  SEM images of NIH 3T3 fibroblasts seeded nanofibers at 24 h (a1, b1 and c1) and 48 h (a2, b2 and c2) a) PCL Nanofiber b) Aminolysed PCL nanofibers and c) Collagen crosslinked PCL nanofibers
3T3 fibroblasts proliferate in all the three scaffolds but the degree of proliferation varies based on the modification of nanofibers. The growth of NIH 3T3 fibroblasts in the case of PCL nanofibers were observed only on the surface. The cells did not appear to infiltrate into the PCL nanofibers. This may be due to hydrophobicity of the PCL nanofibers. APN and CCPN showed increased proliferation and infiltration of NIH 3T3 fibroblasts in the scaffold. Both the scaffolds exhibited increased hydrophilicity observed from water contact angle measurements. Thus, hydrophilicity is hypothesized to increase the biocompatibility and infiltration of NIH 3T3 fibroblasts in PCL nanofibers. The improved infiltration forms multiple-cellular layers, which are expected to induce better tissue formation with homogeneity in cell growth and ECM generation.

Earlier studies also show that collagen immobilisation on the surface of scaffolds improves the hydrophilicity and biocompatibility (Bisson et al 2002, Yang et al 2002, Ma et al 2005a, Duan et al 2007). Increased proliferation and spreading of NIH 3T3 fibroblasts were observed in CCPN in such a way that it covers the entire surface and the layers of nanofibers beneath the surface due to confluence and improved biocompatibility through collagen immobilization. Such growth will facilitate improved tissue regeneration. Through the surface modification strategy like immobilizing collagen, the disadvantages (like cellular attachment and proliferation) of hydrophobic polymers in tissue engineering applications has been overcome.

The proliferation of NIH 3T3 Fibroblast cells on the modified and unmodified PCL nanofibers in vitro was measured by MTT reagent and was plotted in Figure 2.8. On 48th hour, the absorbance intensity of APN(P<0.05) and CCPN(P<0.01) was significantly higher than that of the PCL Nanofibers. The cell proliferation in CCPN did not show any statistical difference than that of APN. However from SEM analysis it is clearly seen that the
proliferation of fibroblast cells are higher in CCPN than APN, and APN better than PCL nanofibres. This indicates that the aminolysis and collagen immobilization significantly improves surface biocompatibility of PCL nanofibers.

Figure 2.8  Cell proliferation of 3T3 fibroblast cells seeded on PCL nanofibers and modified nanofibers (* Indicates significant difference than that of PCL Nanofibers and # indicates significant difference than that of Aminolysed PCL nanofibers, n=3)

These findings suggest that the collagen crosslinked onto the PCL nanofibers can be used as novel functional biomimetic nanofibers toward achieving excellent integration between cells and scaffolds for tissue engineering applications.