Chapter 3

Electrospun Collagen Maintains the Ultra Structural integrity of Triple Helices

3.1 Introduction

Collagen is a natural extracellular matrix (ECM) component of various types of tissues, such as hard bone, thin skin, soft tendon, ligament and other connective tissues. At present, 29 distinct collagen types have been characterized out of which only few types are used to produce collagen based medical devices for tissue engineering. Type I collagen is present in every tissue and supports the mechanical function of the specified organ. The fibrous texture of collagen and their association with the microenvironment decides the mechanical or tensile property and provide structural support to the tissues of the organ. The fibrillar collagen classified as type I, found in bone, lung, skin, cornea, tendon, and the vasculature, type II, which has more specificity towards distribution in cartilage, type III, found in elastic tissues such as embryonic skin, lung and blood vessels, type V is relatively less and found in association with collagen type I in cornea and cartilage contains type XI in association with type II. Body contains 80% of type I, II and III, fairly similar in all species and quite non immunogenic [1]. Structurally, Type I collagen is fibrous in nature and each fibril has a triple helix configuration which consists of three covalently cross linked, interwined polyproline peptide chains. Each collagen fibril molecule consists of three polypeptide chain called α chain which forms triple helix configuration with covalent crosslinkage. When α chains are identical referred as homotrimeric and when they are genetically distinct are referred as
heterotrimer. Type I collagen fibrils are composed of two α1 chains and one α2 chain. The triple helix measures approximately 300nm and the width is 1.5nm. The long triple helical central region in each α chain containing continuous (Glycine –X–Y) n repeat of amino acid motif and X and Y referred to any amino acid. The glycine residues buried inside the core of protein and X and Y exposed on the surface. Where n is the number of repeats of glycine, X and Y varies between 337 – 343 repeats according to the type of collagen. The triple helical region is flanked by telopeptides referred as non helical region also known as crosslink peptides forms about 20 residues at both N and C terminals. This protein motif is the essential condition to identify as a collagen. The specific binding sites of c-terminal telopeptides favour the formation of mature fibrils through its interaction with triple helical monomers. The stability of the collagen fibrils enhanced through the cross-link between the lysine side chains of the telopeptide with the subsequent fibril. The arrangement of triple helix repeats forms the alternating overlap and gap zones possess a particular packing arrangement as a consequence of which it forms mature collagen fibrils possessing a high degree of alignment indicating the repeat of bands named as D periodicity of 67nm, i.e. the collagen molecule staggered by about 22% of their length (67nm) over the nearest neighbour. The periodicity distance D (67nm) is formed by the hole or gap (47nm in length) and overlap zone (20nm in length) determined using high voltage electron microscopic tomography considered as the finger print of fibrous collagens [2, 3, 4]

Fibrous architectural collagen favours the cellular components an optimum microenvironment for structural support which further determines the tensile nature of the native tissue. Therefore collagen has been widely used as a biomaterial to make scaffold to

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create an ideal tissue construct for potential application in the field of tissue regenerative medicine. The advancement in the analytical and purification methods made convenient to produce collagen with low immunogenicity. [5] The supporting features of collagen as a biomaterial are highly biodegradable, good tensile strength and modulus, and low antigenicity. [6, 7] The ideal method to develop the scaffold is electrospinning due to its creation of 3Dimensional fibrous matrix which resembles the native extra cellular matrix. [8] The mechanism of electrospinning nanofibers and the importance of solvent chosen for the process have been explained thoroughly in chapter II. The solvent plays a critical role in the spinnability of the polymer and the morphology of the electrospun fiber. Solvent miscibility with polymer should be homogenous. Extremely volatile solvents with dielectric constant are essential to separate fibers in the process of electrospinning before reaching the target collector. The molecular weight and the concentration of the polymer should be optimized to obtain appropriate viscosity, surface tension and the conductivity of the prepared solution. The applied electric field, flow rate of the polymer and the collecting distance are optimized together to obtain smooth nanofibers [9, 10]. Solubility of collagen is possible with 1,1,1,3,3,3- hexafluoropropan-2-ol (HFP) and 2,2,2-trifluoroethanol (TFE) and are commonly used solvent for the electrospinning of collagen fibres because of its high volatility and less affinity to the collagen in contrast to acid which has high affinity and less evaporation[11,12]. However, it has been reported that fluoroalcohols can lead to conformational change of native proteins. The hydrophobic and hydrophilic interaction of hydrogen bonds maintains the triple helix structure. The collagen solubilisation with fluoroalcohols interacts with hydrophobic and hydrophilic bonds which lead to the separation of the triple helixes and thereby losing the
crystalline structure of collagen. Complete unfold of collagen with fluorinated solvent has been confirmed by Burck et al. [13]. The resultant electrospun matrices do not possess the D periodicity of 67nm banding characteristic of native collagen. Electrospun collagen using fluoroalcohols are found to dissolve readily in aqueous solutions. The originally water insoluble collagen fibril gives rise to a denatured collagen as a water-soluble degradation product and this implies that the water solubility of the electrospun collagen scaffolds is due to a possible conformational change in collagen structure [14].

Fluroalcohols are commonly used to collagen electrospinning because of its role in solubilizing the collagen. However, fluoroalcohols are corrosive and also expensive. Thus, the major challenge in producing electrospun collagen is to use an appropriate solvent system that does not denature or alter the biologically favourable properties of collagen [15].

In selection of solvents, environmental and toxicological factors should also be considered. Recently, there has been a tendency towards the use of solvents that are more benign to humans and to the environment. In this study several combinations of solvents has been studied to dissolve collagen either with toxic and benign solvents in order to reduce or overcome the toxicity in the process of electrospinning. Complete solubility of collagen has been achieved when trifluoroacetic acid is used as part of the solvent combinations. Complete immiscible of collagen was observed in chloroform, dichloromethane and isopropanol were used in the preparation of solvent combinations. Almost 90% of collagen was found to be dissolved in 17 solvent combinations and the remaining undissolved particles could be collagen debris like glycoproteins, etc. (Table -3.1) Acetic acid and DMSO combination has been chosen as solvent combination for collagen electrospinning in the current work. Further
20 more novel combinations were identified in the current research work which can be potentially used for collagen electrospinning.

Environmentally benign solvent, acetic acid has been used in extraction of collagen from various sources without any denaturation and the stability provided through cross linking. Subsequently, it was found that acetic acid alone when used to dissolve collagen did not result in collagen fiber formation. Collagen type I has been electrospun in combination with carrier polymers such as polycaprolactone (PCL) [16] and polyethylene oxide (PEO) [17]. Dong et al have reported collagen can be electrospun in a benign solvent combination of water/alcohol/ salt with shrinkage of matrix while cross linking [18]. Buttafoco and colleagues have shown that fibers are generated from 1-2% collagen in weak acidic solutions only with the addition of copolymers of high molecular weight, which increase the viscosity of the solution and lead to formation of stable jets [19]. The current research work is to fabricate electrospun collagen fibrous matrices without carrier polymer using benign solvent system, which maintains the ultra structural integrity of triple helical, possessing a high degree of axial alignment by exhibiting the characteristic D periodicity banding of 67nm through transmission electron micrography. The developed 3D collagen nanofiber scaffolds were characterized with tensile property suitable modulus for cardiac tissue and its biocompatible, favoured the adhesion and maintenance of primary neonatal cardiomyocytes with expression of contractile proteins.
Novel solvent combinations

<table>
<thead>
<tr>
<th>S No</th>
<th>Solvents</th>
<th>Ratio</th>
<th>Collagen Solubility 2mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GAA : Chloroform : ethanol</td>
<td>2:1:1</td>
<td>0%</td>
</tr>
<tr>
<td>2</td>
<td>GAA : chloroform : methanol : DMSO</td>
<td>4.5 : 2.5 : 2.5 : 0.5</td>
<td>90%</td>
</tr>
<tr>
<td>3</td>
<td>GAA : chloroform : ethanol : DMSO</td>
<td>4.6 : 2.35 : 2.35 : 0.7</td>
<td>90%</td>
</tr>
<tr>
<td>4</td>
<td>GAA : THF : DMSO</td>
<td>7:1:2</td>
<td>90%</td>
</tr>
<tr>
<td>5</td>
<td>GAA : DCM : DMSO</td>
<td>7:1:2</td>
<td>90%</td>
</tr>
<tr>
<td>6</td>
<td>GAA : Acetone : Ethanol : DMSO</td>
<td>7:1:1:1</td>
<td>90%</td>
</tr>
<tr>
<td>7</td>
<td>GAA : EA : DMSO</td>
<td>7:1:2</td>
<td>90%</td>
</tr>
<tr>
<td>8</td>
<td>GAA : DMSO</td>
<td>93:7</td>
<td>90%</td>
</tr>
<tr>
<td>9</td>
<td>Trifluoroacetic acid</td>
<td>1</td>
<td>90%</td>
</tr>
<tr>
<td>10</td>
<td>GAA : DMF : DMSO : 2ethoxyethanol</td>
<td>7:1:1:1</td>
<td>90%</td>
</tr>
<tr>
<td>11</td>
<td>GAA : DMF : DMSO : 2methoxyethanol</td>
<td>7:1:1:1</td>
<td>90%</td>
</tr>
<tr>
<td>12</td>
<td>GAA : Acetone : isopropanol : DMSO</td>
<td>7:1:1:1</td>
<td>0%</td>
</tr>
<tr>
<td>13</td>
<td>GAA : Acetone : methanol : DMSO</td>
<td>7:1:1:1</td>
<td>90%</td>
</tr>
<tr>
<td>14</td>
<td>GAA : THF : DMSO : 2methoxyethanol</td>
<td>7:1:1:1</td>
<td>90%</td>
</tr>
<tr>
<td>15</td>
<td>GAA : DCM : DMSO : 2ethoxyethanol</td>
<td>7:1:1:1</td>
<td>0%</td>
</tr>
<tr>
<td>16</td>
<td>GAA : DCM : DMSO : 2methoxyethanol</td>
<td>7:1:1:1</td>
<td>90%</td>
</tr>
<tr>
<td>17</td>
<td>GAA : EA : DMSO : 2methoxyethanol</td>
<td>7:1:1:1</td>
<td>90%</td>
</tr>
<tr>
<td>18</td>
<td>(10X PBS: Ethanol) : ( TFA :methanol)</td>
<td>(1:1) : (1:1)</td>
<td>100%</td>
</tr>
<tr>
<td>19</td>
<td>(10X PBS: methanol) : ( TFA :ethanol)</td>
<td>(1:1) : (1:1)</td>
<td>100%</td>
</tr>
<tr>
<td>20</td>
<td>(10X PBS: methanol) : (TFA:methanol)</td>
<td>(1:1) : (1:1)</td>
<td>100%</td>
</tr>
<tr>
<td>21</td>
<td>(10X PBS: isopropanol) : (TFA: Methanol)</td>
<td>(1:1) : (1:1)</td>
<td>90%</td>
</tr>
<tr>
<td>22</td>
<td>(10X PBS: Ethanol) : ( TFA : Ethanol)</td>
<td>(1:1) : (1:1)</td>
<td>90%</td>
</tr>
<tr>
<td>23</td>
<td>(10X PBS: methanol) : ( TFA : isopropanol)</td>
<td>(1:1) : (1:1)</td>
<td>100%</td>
</tr>
<tr>
<td>24</td>
<td>TFA : chloroform : ethanol : DMSO</td>
<td>450 : 250 : 250 : 50</td>
<td>0%</td>
</tr>
</tbody>
</table>

Table 3.1 Novel solvent combinations to dissolve collagen for electrospinning. The highlighted combinations does not solubilize collagen.
3.2 Materials and Methodology

Collagen Type I of Fish origin, commercially known as Helisorb was obtained from Eucare Pharmaceuticals, Chennai. Solvents acetic acid and Dimethylsulfoxide (DMSO) were analytical grade and obtained from SRL, Mumbai and Merck, USA.

3.2.1 Electrospinning of collagen

A custom built electrospinning setup consisting of a stabilized high voltage power supply, Sri Ramachandra University, Chennai; a programmable peristaltic pump Ravels Tech, Chennai; silicone tubing with a 26-G needle and a grounded metal plate as a stationary collector were used to generate nanofibers as described in chapter 2. Collagen type 1 was dissolved in Glacial acetic acid: DMSO at a ratio of 93:7 to prepare a 10% weight/volume solution by overnight agitation. Nanofibers were generated at a constant voltage of 17 KV and the collagen solution was supplied to the 26 G needle at a flow rate of 0.60 ml/h. The working distance between the needle and the collector was 23 cm. Fibers were collected for defined durations and the matrices were cross-linked with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, (EDC, SRL Mumbai) to stabilize collagen fibers [20]

3.2.2 Electron microscopy

Characterization of the collagen nanofibers was carried out by Scanning Electron Microscopy (S-3400N). The cross linked scaffolds were carefully cut into 1 cm square mats and the samples were coated with gold using a sputter coater. Multiple digital images of three different scaffolds were taken and the average fiber diameter was determined by taking the average of 30 measurements chosen at random across each image set. Digital images were
analyzed using Image J software. For Transmission Electron Microscopy (TEM), the scaffolds were fixed with 3% glutaradehyde using standard protocols, rinsed in cacodylate buffer and post-fixed in 1% osmium tetroxide for 1 hour and embedded with epoxy resin. Ultrathin sections were examined with a Philips Tecnai T12 Instrument.

3.2.3 Porosity measurement
The porosity of the collagen nanofiber mats was analyzed by examining the SEM images using Image J software as described previously [21]. The Three upper, middle and lower layers of nanofibrous membrane were obtained by adjusting thresholds of SEM image. The original 256 gray scale image was first converted to binary image to obtain a total number of pixels (N) in the binary image. Also the number of white pixel (n) in each layer of binary images is noted to calculate the porosity percentage of each layer using the formula: \( P = (1 - n/N) \times 100 \). The average pore size of each layer (\( P_1, P_2, \) and \( P_3 \)) gives the total porosity of the membrane. Four SEM images of magnifications of 2000 and 5000 were analyzed for \( P_1, P_2 \) and \( P_3 \) and the overall percentage of porosity was estimated by calculating the mean and standard deviation.

3.2.4 Tensile property
Cross-linked collagen scaffolds that had been collected for 8 hours were cut into strips with dimensions of 3 cm in length and 1.5 cm in width and were roughly 0.3mm in thickness. Tensile strength measurement of the electrospun collagen matrices was carried out using a universal tensile tester, INSTRON 3365 with a 10N load cell and performed at a speed of 1 mm/min [22] A total of 4 samples were used for the analysis to calculate the mean and standard deviation.
3.2.5 Cell culture using L6 cell line

Collagen nanofibrous scaffolds were cut into 1 cm squares, washed with sterile phosphate buffered saline (PBS) followed by sterilization with serial dilutions of ethanol and stored in PBS supplemented with antibiotics at 4 degree C. Rat skeletal myoblasts (L6 cell line) were cultured in DMEM high glucose medium supplemented with 10% fetal bovine serum and antibiotics. Myoblasts were seeded onto scaffolds in non-adherent culture dishes at a seeding density of 2.5×10^4 cells/scaffold and incubated at 37° C in an atmosphere containing 5% carbon dioxide. The culture medium was refreshed every 2 days. The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) test assesses the cytotoxicity by measuring the cell viability and proliferation. [23]. The scaffolds were tested for MTT assay at different time point of 2, 4, and 6 days and the color development measured at 595 nm using a spectrophotometer. The mean and standard deviation for 3 scaffolds per time point were calculated to determine cell viability and proliferation on the scaffolds.

3.2.6 Primary cardiomyocytes culture

The guidelines followed as per the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and was officially permitted by the Institutional Animal Ethics Committee of Sri Ramachandra University (study no: IAEC/XXII/SRU/174/2011). Culture of primary cardiomyocytes from 2-3 days old Sprague-dawley rats was performed as described earlier [24]. Briefly, the hearts of the neonatal rats of total 10 numbers were chopped and subjected to trypsin digestion and the isolated cardiomyocytes were pooled together prior to seeding. The scaffolds were seeded with NRVCM in nonadherent culture dishes at 5×10^5 cells/scaffold and incubated at 37° C with 5% carbon dioxide. Seeded with
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1x10^5 cells/per cover glass was coated with 2.5% gelatin solution for 2D culture. The culture medium (DMEM: F12 in the ratio of 4:1) was changed at intervals 2 days. Microscopic examinations of 10 fields in three different scaffolds were observed to count the average no of beats per 2 minutes. The scaffolds were examined at 3, 5, 7, 9, 11, 14 and 17 days time point. The images were captured with a Nikon TE2000 Eclipse inverted microscope possesses with CCD camera captured 30 frames per second to convert into video with Image Pro Software.

3.2.7 Immunocytochemistry and confocal microscopy
The cell-seeded scaffolds were cryopreserved at defined time points of the assays. Immuno staining of cryosections was carried out as described previously [24]. Briefly, thin sections were fixed in 3.7% formaldehyde, permeabilised with triton X 100, after addition of primary antibody, muscle specific actin and desmin, (both from Biogenex, USA). The sections were incubated, washed and stained with Goat anti Mouse FITC (Merck), counter stained with propidium iodide to emphasize the nuclei and analyzed on a Zeiss LSM 510 Laser Scanning Confocal Microscope.

3.2.8 Reverse transcription polymerase chain reaction (RT-PCR)
RNA plus (MP biological) was used to isolate the total RNA and performed according to the instructions and Verso cDNA synthesis kit (Abgene) was used for cDNA synthesis. PCR steps includes denaturation, annealing and extension of 94°C for 30 seconds, 60°C/63°C for 30 seconds, 72°C for 30 seconds, for 25/29 cycles, GAPDH / desmin respectively. Primer with product size, 556bp for GAPDH 5’-ACC ACA GTC CAT GCC ATC-3’ (forward) and
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5’-TCC ACC ACC CTG TTG CTG-3’ (reverse) and with 224bp for desmin 5’-CAA CCT TCC GAT CCA GAC CT-3’ (forward) and 5’-GAG TGG AAA AGG CTG GCT TC-3’ (reverse) has been used for PCR analysis.

Statistical analysis
All numerical data are expressed as Mean ± standard deviation (SD).

3.3 Results

3.3.1 Physical characterization of collagen fibrous matrix
SEM image analysis revealed the deposition of fibers in a random manner (Figure 3.1) onto the stationary collector with the average fiber diameters in the range from 200 nm to 1100nm (Figure 3.2). The collagen fibrous matrices also exhibited a porosity percentage of 44.3 ± 1.79, when measured as described by Ghasemi-Mobarakeh et al (Table 3.2). Recent studies have also shown that fluroalcohols commonly used to electrospin collagen type 1 lead to a denaturation of the collagen where the ultra structural integrity of triple helix repeats in the form of 67nm D periodicity banding pattern characteristic to native collagen is no longer present in transmission electron microscopy (TEM) [13]. We have successfully electrospun collagen type I nanofibrous scaffolds using the binary benign solvent and were stabilized successfully using EDC to support contracting cardiomyocytes for long term survival in static culture without influence of external electrical stimuli. The matrices stored in phosphate buffered saline (PBS) for 2 months were used to examine TEM. Ultrathin sections of scaffolds revealed the presence of the 67nm D periodicity banding pattern characteristic feature of native collagen (Figure 3.3). The TEM images prove that the native properties of collagen were maintained after electrospinning in glacial acetic acid and DMSO is the

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highlight of the study. The mechanical properties of the scaffolds were optimal to withstand the forces of the contracting cardiomyocytes. Using uniaxial tensile measurement apparatus, measured the Young’s modulus, elongation at break, and tensile strength of the nanofibrous scaffolds (Table 3.3). The obtained modulus of 3.45 ± 1.66 kPa, a tensile strength of 1.57 ± 0.41 kPa and an elongation of 95.2 ± 28.38 %, render them suitable for contracting cardiomyocytes.

**Scanning electron microscopy**

*Figure 3.1* A represents the electrospun collagen nanofibrous sheet stored in PBS. B represents the SEM image of electrospun collagen type I. The matrix illustrates the porous, fibrous nature of the scaffold. Scale bar = 20µm
Transmission electron microscopy

Figure 3.2 Transmission electron micrographs of electrospun collagen nanofibrous scaffold exhibit the triple helical repeats of 67nm D periodicity banding pattern typical of native collagen (inserted scale bar 250 nm and 50 nm.)
Porosity Measurement of binary images of different samples with various thresholds

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>Magnification</th>
<th>Top layer (P1)</th>
<th>Middle layer (P2)</th>
<th>Lower layer (P3)</th>
<th>Average porosity percentage</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>2000</td>
<td>75.0</td>
<td>39.0</td>
<td>19.2</td>
<td>44.4</td>
</tr>
<tr>
<td>2</td>
<td>2000</td>
<td>74.0</td>
<td>44.0</td>
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<td>46.6</td>
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<tr>
<td>3</td>
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<tr>
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<tr>
<td>5</td>
<td>5000</td>
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<td>43.8</td>
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<tr>
<td>6</td>
<td>5000</td>
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<td>8</td>
<td>5000</td>
<td>63</td>
<td>43</td>
<td>21</td>
<td>42.3</td>
</tr>
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</table>

Table-3.2 Represents the porosity measurements of SEM images of four different scaffolds (n=4) at two different magnifications using image J software. Porosity percentages of three thresholds that help in distinguishing top, middle and lower layers of the nanofibers mat were measured as $P_1$, $P_2$ and $P_3$. Total porosity percentage was the average of the three and the overall percentage of porosity was estimated by calculating the mean and standard deviation.
Figure 3.3 Graphical representation of distribution of fiber diameters measured from SEM images of four independent scaffolds (n=4) using image J software. The fiber diameter ranges from 200nm-1100nm.
Mechanical property of collagen nanofiber scaffold

<table>
<thead>
<tr>
<th>Sample</th>
<th>Maximum Load (kPa)</th>
<th>Tensile stress at maximum load (kPa)</th>
<th>Tensile strain at maximum load (%)</th>
<th>Modulus (kPa)</th>
</tr>
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<tbody>
<tr>
<td>C1</td>
<td>0.01</td>
<td>1.13</td>
<td>120.08</td>
<td>1.71</td>
</tr>
<tr>
<td>C2</td>
<td>0.01</td>
<td>1.35</td>
<td>91.92</td>
<td>2.65</td>
</tr>
<tr>
<td>C3</td>
<td>0.01</td>
<td>1.58</td>
<td>50.14</td>
<td>6.18</td>
</tr>
<tr>
<td>C4</td>
<td>0.01</td>
<td>2.23</td>
<td>118.94</td>
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</tr>
<tr>
<td>Mean</td>
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<tr>
<td>Standard deviation</td>
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<td>0.41</td>
<td>28.38</td>
<td>1.66</td>
</tr>
</tbody>
</table>

Table-3.3 The tabular column represents the mean and standard deviation of tensile strength, stress and young's modulus of 4 different samples of collagen nanofibrous membrane.

3.3.2. Biocompatibility studies with cell lines and primary cardiomyocytes cultures

Studied biocompatibility of the electrospun scaffolds using cell lines. Using the MTT assay, proliferation of L6 rat skeletal myoblasts at varied time points were analyzed. Observed that the OD values after two days in culture on the nanofiber scaffolds were 1.59 ± 0.17 and increased at later time points with 2.41± 0.04 on day 4 and 2.53±0.25 on day 6 respectively (Figure 3.4). Scaffolds cultured with myoblasts for six days were cryosectioned and examined for muscle specific proteins using immunofluorescence staining. Confocal immunofluorescence analysis revealed a prominent expression of desmin and muscle specific Actin, throughout the matrices (Figure 3.5), indicating the suitability of the scaffold for muscle cells. Also characterized the scaffolds for primary ventricular cardiomyocytes from...
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neonatal rats (NRVCM) and seeded onto 3D collagen scaffolds to study them for cardiac tissue engineering applications. Two days post seeding, NRVCMs adhered well onto flat culture ware and 3D collagen scaffolds and organized themselves into groups of contracting cells that began to beat synchronously by day three (see supplementary Video). On observation of the contracting cells under the microscope revealed that the number of beats per 2 minutes was initially lower for 3D scaffolds with 120 ± 3.9 and 92 ± 16 beats for day 3 and day 7, when compared to 2D cultures showed 200 ± 8.3 and 250 ± 12.9 respectively (Figure 6). The initial difference in beating frequency could be due to cell acclimatize to adhere, communicate and organize on a 3D scaffolds. 2D cultures continued to beat well on day 9 with 250±7 but after ten days the beating frequency was drastically reduced in 2D cultures. The contractility of cardiomyocytes on 11 and 14 days showed 170±1.4 and 174.2 ± 2, and no beating was observed on day 17. In contrast, cardiomyocytes on 3D collagen scaffolds showed contractility with 300 ± 1.6, 310 ± 5.7, 297.1 ± 9.7 and 301 ± 3.6 beats on days 9, 11, 14 and 17 respectively (Figure 3.6). Confocal immunofluorescence analysis of cardiac marker Troponin T revealed a strong expression on 3D scaffolds with different time point of post seeding (Figure 3.7). Also the expression of desmin using Reverse Transcription Polymerase Chain Reaction (RT-PCR) on all time points has been studied. Briefly, 100 ng of cDNA of each sample were amplified using primers for desmin and GAPDH as described. We observed that there was a reduction in expression levels of desmin in cardiomyocytes cultured on 2D surfaces on day 14 post seeding when compared with day 7 (Figure 3.8), which is in accordance with the beating data. The cardiomyocytes that are grown on 3D collagen scaffolds had sustained expression of desmin on day 7 and 14, when
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compared with the 2D surfaces, which also corroborates our beating data. This work indicates that electrospinning collagen using glacial acetic acid and DMSO can generate favourable 3D nanofibrous scaffolds for both cell line and primary cultures, hence suitable for tissue engineering applications preferably optimal for contracting cardiomyocytes.

**Cell viability assay**

![MTT Assay](image)

*Figure 3.4 Biocompatibility of the collagen scaffold evaluated by MTT assay using L6 rat skeletal myoblast, cell growth tested at 3 different time points (2, 4, 6, days in culture). Myoblasts proliferated well on collagen nanofibers scaffolds.*

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Actin and desmin expression studied by confocal microscopy on collagen scaffold

Figure 3.5 Rat skeletal myoblast (Cell Line) were seeded on collagen nanofibrous scaffold and cultured for 6 days, cryosectioned and stained. Collagen matrices showed prominent cell adherence and proliferation throughout when examined by immunofluorescence staining using confocal microscopy. A. anti-muscle specific actin FITC, B. Nuclear staining with propidium iodide, and C. Merged image, D. anti-desmin FITC, E. nuclear staining with propidium iodide and F. merged image. Scale bar = 50µm

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Graphical representation of mean and standard deviation of contracting cardiomyocytes

Figure 3.6 Graphical representation of beating (contracting) of cardiomyocytes on 2D tissue culture ware versus 3D collagen scaffolds on designated time points.
Troponin T expression studied by confocal microscopy on collagen scaffold

Figure 3.7 Cryosections of collagen nanofiber scaffolds seeded with (Primary cells) neonatal rat cardiomyocytes were stained with cardiac specific marker troponin T and examined by immunofluorescence confocal microscopy. A, D, G: anti-troponin-FITC, B, E, H: phase contrast, and C, F, I: merged images. Images A to C (7 day time point), D to F (14 day time point) and G to I (17 day time point) respectively. Scale bar = 50µm
Gene expression study

Figure 3.8 Agarose gel electrophoresis image of RT-PCR analysis of expression of Desmin on day 7 and 14 by cardiomyocytes seeded onto 2D culture ware versus 3D collagen nanofibers scaffold respectively. GAPDH was used as control.

Supplementary Video 1 and 2 Video microscopy of neonatal cardiomyocytes seeded on 2D and 3D culture.
1. Movie clips of beating cardiomyocytes on 2D culture
2. Movie clips of beating cardiomyocytes on 3D culture

3.4 Discussion
Acetic acid is widely used for the extraction of collagens from natural sources and is a solvent that does not denature the favourable properties of collagen. DMSO is known as a co
Electrospinning of collagen type 1 to produce nanofibers has been commonly achieved with fluoroalcohols, which are expensive, toxic to the environment and also denature the native properties of collagen. Weak acetic acid and PBS ethanol based solvents have also not maintained the 67nm D banding property of collagen after electrospinning. The study provide conclusive evidence to show that it is possible to successfully electrospin collagen type 1 alone without the need for carrier polymers using acetic acid and DMSO as a solvent and the work has been filed for patent is definitely cost effective and a more environment friendly alternative to fluoroalcohols. This study is the first to show that the benign binary solvent maintains its molecular structure and favourable properties, which are essential to use as a biomaterial in the field of tissue engineering.

The biocompatibility study showed that the myoblasts proliferated well on the collagen scaffolds by their expression of desmin and actin. The cytoskeleton of muscle cells include proteins whose primary function is to connect, anchor and coordinate structural components such as the myofibrils, mitochondria, nuclei and organelles. It is composed of three integral components; intermediate filaments (IFs), microfilaments and microtubules. Desmin is the major interfilament protein found in skeletal and heart muscle that has a vital role in maintaining the structural integrity of myocytes and contributes to force transmission. Actin forms the microfilaments and is also an integral part of the cytoskeleton and contractile apparatus. Engler and co-workers have shown that the elasticity and rigidity of scaffolds directly affects the maintenance and differentiation of cells. Neonatal ventricular...
cardiomyocytes cultured on substrates of ~10 kPa characterized the alignment of sarcomeres [30]. The mechanical property analysis data proved that the tensile strength appears relatively nearer to native cardiac tissue measures between 3–15 kPa [31] and also Engler et al has shown contracting cardiomyocytes on hydrogels with matrix stiffness of 1kPa. Scaffolds developed with low modulus can be potentially used also in soft tissue engineering [32]. The collagen scaffolds developed with these features favour cardiomyocytes to maintain their contractility for three weeks, which was not so in the case of 2D culture. This static 3D cardiac model can also be further improved by incorporating electrical stimulation strategies for prolonged studies in drug discovery. The results of the study show that collagen type 1 can be electrospun using a simple benign solvent system. The collagen nanofiber maintains its native integrity and is a promising candidate for providing the support and microenvironment required for cardiac tissue-engineered constructs in vitro.
3.5 Conclusion

Thus, the electrospinning of collagen with a benign solvent combination can be utilized to reduce the cost of producing electrospun collagen scaffolds that preserve the native integrity and can be potentially applied for tissue engineering applications. The developed collagen scaffold was also characterized to support contracting cardiomyocytes in vitro, which can be further developed as in vitro 3D models for applications in cardiac tissue engineering. These collagen scaffolds developed, along with stem cell technology, are considered to be a potential source in the fields of tissue engineering and regenerative medicine.
3.6 References


