

# PROGRESS REPORT

<b>1. Project Title</b> Cell sheet engineering on electrospun scaffolds for efficient cell supply in skin tissue engineering.	<b>DST No:</b> SR/FT/LS-128/2009 21-12-2011
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<b>3. Co-PI (Name &amp; Address):</b> NA	<b>Date of Birth:</b> NA
<b>4. Broad area of Research : Life Sciences</b>	
<b>4.1 Sub Area : Tissue Engineering, Cell Biology</b>	
<b>5. Approved Objectives of the Proposal:</b> <b>Year I</b> <ul style="list-style-type: none"> <li>• Fabrication of polymer scaffold by electrospinning</li> <li>• Synthesis of thermoresponsive culture surface</li> <li>• Characterization of scaffolds</li> <li>• Cell culture study using cell line on polymer scaffolds</li> <li>• Isolation and characterization of primary cells</li> </ul> <b>Year II</b> <ul style="list-style-type: none"> <li>• Cell culture study using cell line on polymer scaffolds</li> <li>• Isolation and characterization of primary cells</li> </ul> <b>Year III</b> <ul style="list-style-type: none"> <li>• Cell culture on polymer scaffolds</li> <li>• Construction of in vitro skin structure by monolayer transfer</li> </ul>	
<b>Date of Start</b> :19-01-2012	<b>Total cost of Project</b> : 19.44 lakhs
<b>Date of completion</b> :18-01-2015	<b>Expenditure as on</b> : 31-08-2014

## 6. Methodology

### 6.1 Materials used

Polycaprolactone (PCL), Chitosan (CS) (95% deacetylated), Acetone, Formic acid, IMDM, Panscerin 801 + growth factors, Collagen type-I, Paraformaldehyde, Triton X-100, Primary antibody- Keratin-10, Involucrin, Secondary antibody- FITC, TRITC, Ethanol, PBS, Dispase II, Trypsin 10X, Betadiene, Cell strainer 70 um, 10 ml syringe, 0.22um filter, Fluorescein di acetate, propidium iodide, tetra hydrofuran, dimethylsulphoxide, Keratin 14, Keratin 1.

### 6.2 Fabrication of Chitosan scaffold by electrospinning

The electrospinning system included a syringe, 22-gauge stainless-steel needle, a syringe pump, high-voltage power supply, a spinning drum as the collector. The syringe was mounted horizontally on the syringe pump, and spinning solution was drawn from the needle tip with an electrostatic force generated from the high voltage applied between the tip and the collector. The applied voltage and flow rate were controlled. CS-PCL solution 2:8 v/v ratio was prepared by overnight stirring of Chitosan and PCL solutions. The solution was filtered twice by passing through a gauze cloth and stored at 4°C until use.

The electrospinning of chitosan require stringent conditions of high voltage and high polymer concentration. High voltage to syringe pump can interfere in the performance of the system and the flow rate of polymer solution. Hence the syringe pump was modified with the help of the supplier by adjusting the height and addition insulation to the unit and the wiring system. The parameters such as flow rate, Tip to collector distance (TCD), applied voltage and the speed of mandrel were optimized: Voltage-16.0 kV/cm, Flow rate-1.0 ml/h, TCD-15 cm

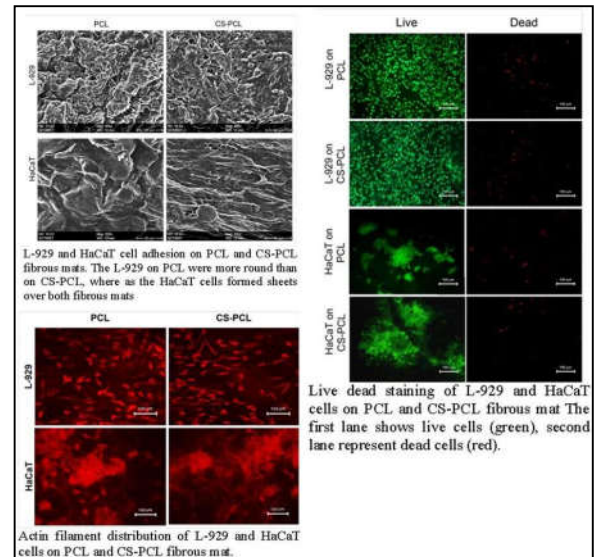
### Characterization of electrospun scaffolds

Since PCL is an already reported material for elctrospinning, the newly fabricated CS-PCL mats were compared with PCL. SEM analysis showed bead free uniform fibers at nano to micro meter diameter range.

The chemical structure and possible interactions between the polymers components in the blend was identified by FTIR. The IR spectrum of PCL and CS-PCL electrospun mats and the pure chitosan were obtained. The spectral scans of the CS-PCL scaffold were recorded at a resolution of 4 cm<sup>-1</sup> in the range from 4000 to 400 cm<sup>-1</sup> and compared with PCL mat and CS powder. The CS showed characteristic peaks at 3431 cm<sup>-1</sup> for N-H and O-H stretching and peak at 2877 cm<sup>-1</sup> from asymmetric bending of C-H group. The spectrum of PCL showed peaks at 1720 cm<sup>-1</sup> and 2919 cm<sup>-1</sup> for ester carbonyl group and C-H respectively. The O-H stretching peak for CS at 3431 cm-1 in the CS-PCL scaffold was flattened.

The tensile strength of CS-PCL and PCL fibrous mats were analyzed in a Instron Universal Materials Testing Machine compared with murine skin tissueThe maximum percentage strain expressed by CS-PCL was lower (26.73 %) compared to that of PCL (84.14%) due to decrease in elasticity. However the Young's modulus of CS-PCL (12.41 MPa) was increased compared to PCL (3.94 MPa). Additionally the CS-PCL showed 66.6 % of tensile property of murin skin tissue (18.20 MPa).

General cytotoxicity of scaffolds was carried out using L-929 cells and specific cytocompatibility for skin tissue engineering was assessed using HaCaT cells. The cytotoxicity of PCL and CS-PCL electrospun fibers were tested by direct contact method (ISO10993-5) with L-929 fibroblast monolayer for 24 h showed non-cytotoxicity. The cytotoxicity by elution method followed by MTT assay on L-929 fibroblast cells showed 95.2± 4.8 % activity with PCL extract and 100 ± 4.6 % activity with CS-PCL extract. Cell adhesion and viability of cells on PCL and CS-PCL fibrous scaffold analyzed using L-929 and HaCaT cells showed viability, cell –cell contact and cellular integration with fibrous mats.



HaCaT cells expressed patched cell growth on PCL and CS-PCL. The cytoskeletal staining of L-929 and HaCaT cultured on PCL and CS-PCL showed similar adhesion and spreading. HaCaT cells showed extensive patch formation and characteristic polygonal morphology on CS-PCL. The proliferation of cells on electrospun mats analyzed using L-29 cells and MTT assay confirmed significantly more cell growth on CS-PCL compared to PCL.

### 6.3 Fabrication of modified PCL based scaffold by electrospinning

The electrospinning system included a syringe, 22-gauge stainless-steel needle, a syringe pump, high-voltage power supply, a spinning drum as the collector. The syringe was mounted horizontally on the syringe pump, and spinning solution was drawn from the needle tip with an electrostatic force generated from the high voltage applied between the tip and the collector. The applied voltage and flow rate were optimized 10% PCL solutions was prepared in Formic acid: Acetone mixture and electrospun into fibrous mats.

#### 6.3.1 Fabrication of Porous PCL (PPCL) scaffolds by electrospinning

Fibrous scaffolds have been demonstrated as an ideal substrate for promoting adhesion, proliferation, and differentiation of various types of cultured cells. They act as temporary substitute for the native extracellular matrix and promote self-repair of cells. The mechanical stability, porosity and adequate, surface properties are critical determinants for fabricating a new scaffold. For imparting porosity the solvent system for dissolving PCL was modified. 10% of PCL was prepared by dissolving in THF: DMSO (9:1) and subsequently electro spun to produce fibrous mats. The parameters such as flow rate, Tip to collector distance (TCD), applied voltage and the speed of mandrel were optimized: Voltage-16.50 kV, Flow rate-1.50 ml/h, TCD-12 cm. The optimized parameters were used to fabricate porous scaffolds for subsequent applications.

#### 6.3.2 Modification of electrospun scaffolds.

Biodegradation is a desirable long-term solution for the disposal of used material. Degradation of PCL in a living environment can result from simple chemical hydrolysis of ester bonds or from enzymatic attack. The *in vivo* biodegradation of PCL is a very slow process due to poor bioregulatory activity, high hydrophobicity, lack of functional groups and neutral charge. Two approaches were followed to improve the degradation of PCL – 1) increase surface area by introducing pores into the electrospun fibers and 2) Chemically treat the surface by alkali to introduce hydrophilic groups. Hence the electrospun fibrous mats were modified by both methods and analyzed for faster degradation. PCL mats were fabricated in Formic acid : Acetone solvent where as the Porous PCL (PPCL) mats were fabricated using Tetrahydrofuran : Dimethyl sulfoxide solvent. The electrospun scaffolds of PCL and PPCL were subjected to alkaline hydrolysis. The scaffolds were incubated in 4M NaOH for 1 h with intermittent shaking to facilitate the hydrolysis reaction. The hydrolyzed scaffolds were designated as HPCL and HPPCL.

#### 6.3.3 Characterization of electrospun scaffolds.

The synthesized scaffolds were characterized by SEM, FTIR, water contact angle and Degradation kinetics.

#### 6.3.4 Scanning Electron Microscopy

SEM analysis of PCL and HPCL scaffolds showed beaded appearance. Hence a second batch of polymer solution for PCL was prepared and electro spun to produce bead free fibers (Figure 1). The PPCL and HPPCL mats showed bead free porous fibers. In the HPCL and HPPCL mats fiber breakage due to alkaline hydrolysis was observed.

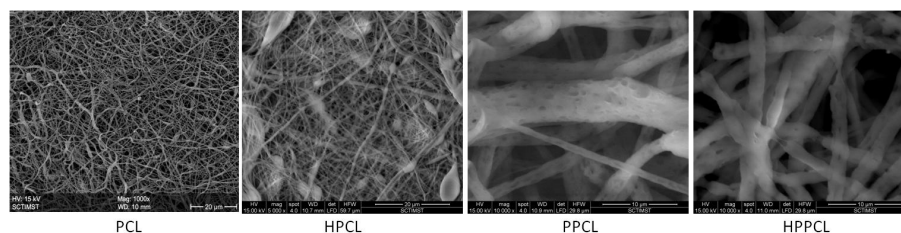


Figure 1 Morphology of electrospun mats under scanning electron microscope. Polycaprolactone (PCL), Hydrolyzed PCL (HPCL), Porous PCL (PPCL) and Hydrolyzed PPCL (HPPCL).

### 6.3.5 Surface characteristics

The surface wettability was determined by analyzing the water contact angle by sessile drop method (Figure 2). When PCL was hydrolyzed, the water contact angle decreased indicating acquiring more hydrophilic moieties. However when PCL with porous fibers were hydrolyzed the water contact angle didn't show any difference. This could be due to the architecture and surface property of HPPCL.

The chemical structure and possible interactions between the polymers components in the blend was identified by FTIR. The IR spectrum of PCL, PPCL, HPCL and HPPCL were obtained. The spectral scans of the scaffold were recorded at a resolution of  $4\text{ cm}^{-1}$  in the range from  $4000$  to  $400\text{ cm}^{-1}$ . The IR spectrum showed peaks at  $1720\text{ cm}^{-1}$  and  $2919\text{ cm}^{-1}$  for ester carbonyl group and C-H respectively. The peaks at  $1632$ ,  $1560\text{--}1530$ ,  $1360$ ,  $1233$ ,  $900\text{ cm}^{-1}$  were found to increase in intensity as a result of the rigid segments being exposed due to alkaline hydrolysis (Figure 3).

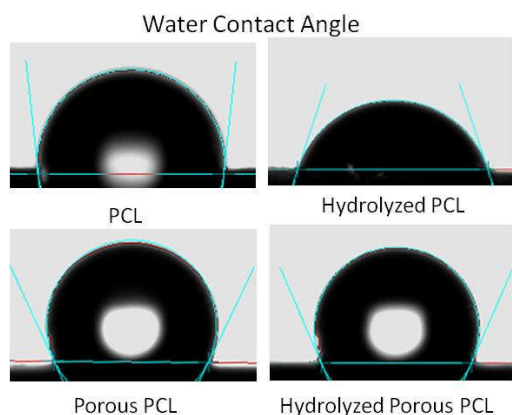


Figure 2 Water contact angle analysis of various modified electrospun mats.

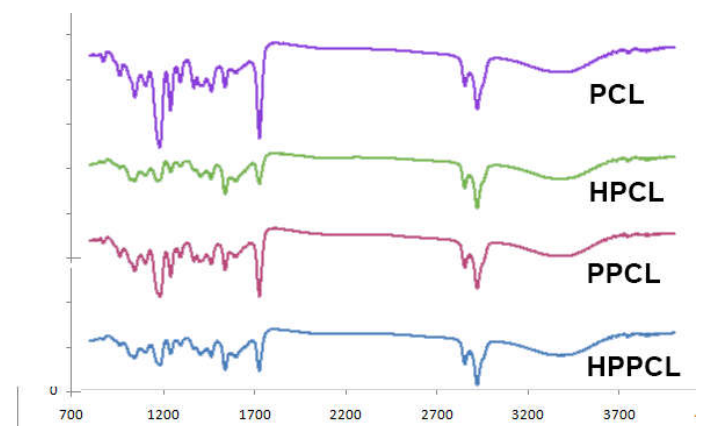


Figure 3 FTIR spectrum of various electrospun mats.

### 6.3.6 *In vitro* degradation of PCL based scaffolds

Substrates for wound care application by tissue engineering methodology require biodegradable biomaterial. New methods of priming biodegradation were tried on electrospun scaffold. The surface area and the surface wettability or functional groups determine degradation properties to a large extent. More over the degradation has to be analyzed in a suitable vehicle with strict aseptic conditions. The *in vitro* degradation of scaffolds was analyzed in two systems. System 1 consisting of Simulated Wound Fluid (SWF) and System 2 consisting of serum free culture medium with  $100\text{ ug/ml}$  Dispase II enzyme. Triplicate samples with known weight and  $2\times 2\text{ cm}$  was immersed in  $5\text{ ml}$  of System 1 and 2 and incubated at  $37\text{ }^{\circ}\text{C}$  for 21 days. At each time points the samples were dried and weighed to analyze degradation. Scaffolds in SWF showed mild swelling as shown by an increase in weight by 7<sup>th</sup> day. Decrease in weight was noted after 7 days suggesting degradation (Figure 4). Similar observation was also noted in scaffolds treated with dispase with an exception on HPCL. This indicates that hydrolysis of electrospun mats could be a simple and efficient method for initiating degradation. This process will prime the scaffolds for degradation before it is applied on host tissue.

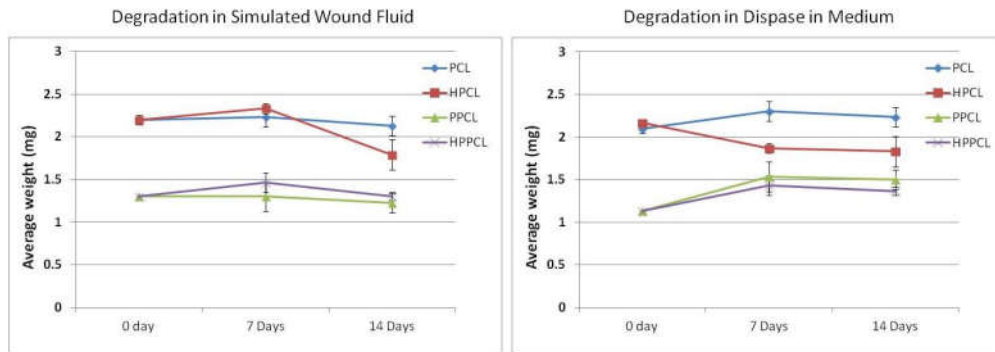


Figure 4 In vitro degradation of various electrospun mats in SWF and dispase protease enzyme.

## 6.4 Cell culture study using cell line on polymer scaffolds

### 6.4.1 Cytotoxicity

General cytotoxicity of scaffolds was carried out using L-929 cells by direct contact method (ISO10993-5). High-density polyethylene and copper discs were used as negative and positive controls respectively. All the scaffolds were found to be non cytotoxic. The scaffolds of size 4mm x 4mm was carefully placed on the cell monolayer and cells were incubated for 24h. Cytotoxicity was assessed by monitoring the morphology, cell detachment, cell lysis and vacuolization of the cells under an inverted phase contrast microscope. The viability of cells after direct contact test was also determined by neutral red staining. The scaffolds in direct contact with L-929 fibroblast monolayer for 24 h showed viability and non-cytotoxicity.

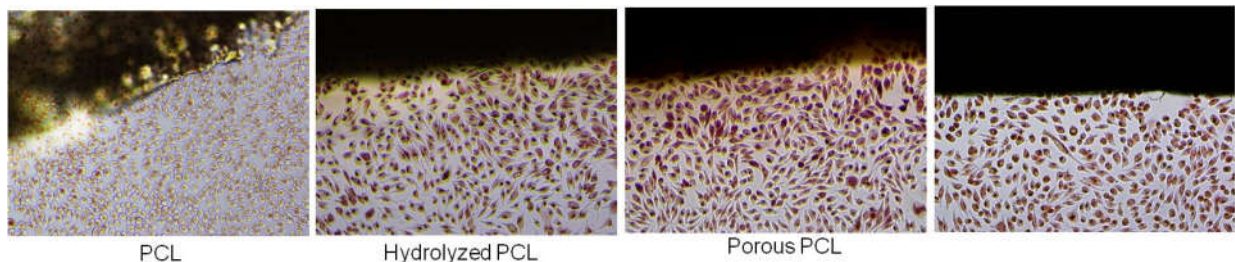


Figure 5 Cytotoxicity and viability analysis of electrospun mats by direct contact method using L-929 cells. Red colour indicates the neutral red uptake by viable cells.

### 6.4.2 Cell adhesion, viability and morphology

Cell adhesion on fibrous scaffolds were analyzed using L-929 cells. The cells adhered on the samples were analyzed for viability its viability using fluorescein di acetate(FDA) and Propidium Iodide (PI) staining (Figure 6). The cell morphology was analyzed by staining actin microfilaments with rhodamine tagged phalloidin . Extensive cell adhesion was noted on PPCL. HPCL and HPPCL scaffolds compared to PCL scaffolds (Figure 7). L-929 cells cultured on scaffolds adhered and spread on the scaffolds.

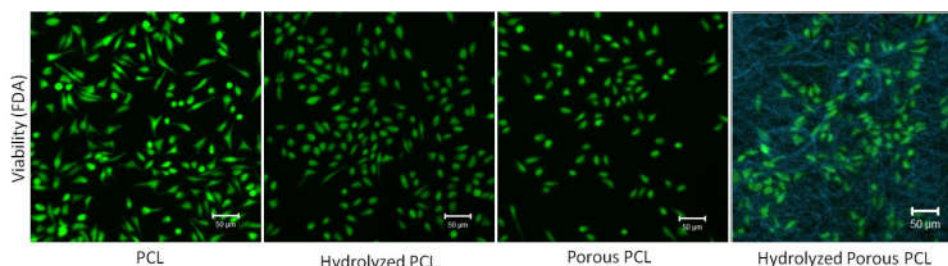


Figure 6 Cell adhesion and viability of L-929 cells on various electrospun mats. Only green fluorescence from FDA was observed without any red fluorescence of PI indicating the cell viability.

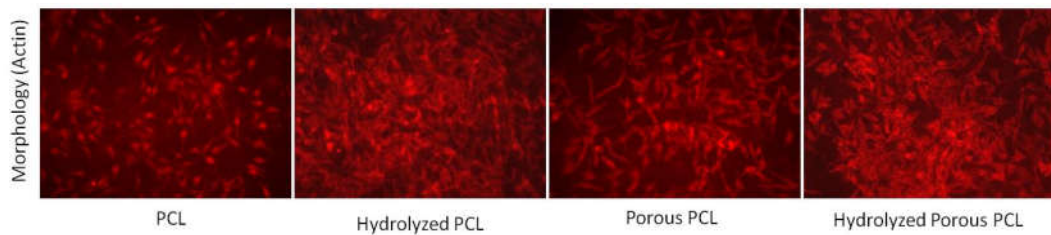


Figure 7 Actin staining of cells adhered on various electrospun mats. Cells expressed characteristic spindle morphology on all materials but the cell adhesion compared was found to be in the order PCL<PPCL<HPCL<HPPCL.

### 6.4.3 Hemocompatibility of scaffolds

The hemocompatibility of the scaffolds were analyzed by measuring hemolysis and platelet adhesion using human blood (ISO 10993-4). The scaffolds were incubated in human blood for 30 mins with continuous shaking. The scaffolds showed no hemolysis with good platelet adhesion suggesting its hemocompatible nature (Figure 8).

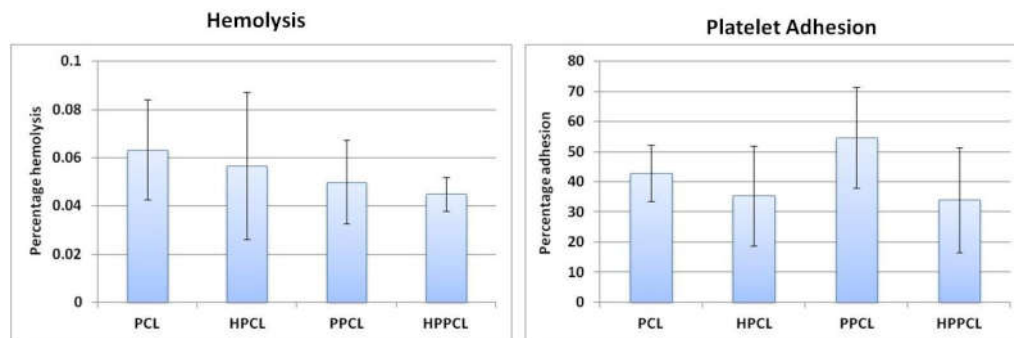


Figure 8 Hemocompatibility analysis of various electrospun mats. The porous PCL mats was found to be less hemolytic and good platelet adhesion indicating suitability as wound dressing matrix.

## 6.5 Fabrication of antibiotic loaded microbead embedded electrospun scaffolds.

### 6.5.1 Microbead preparation.

2% Chitosan solution was prepared by overnight stirring in 0.5M glacial acetic acid. The resultant solution was then filtered twice through a gauze cloth and kept at 4<sup>0</sup>C till use. To a stirred suspension of paraffin oil 2ml of CS was added and stirred for 10 mins at 2500 rpm. 0.5ml of 50% Glutaraldehyde was then added to the stirred suspension and continued stirring for another 20 min. 50 ml of sterile distilled water was then added and the micro beads were filtered through a BD 70 $\mu$  filter and washed with ethanol twice, spread on wattman filter paper and air dried. Antibiotic loaded micro beads were prepared as described above with the polymer to drug concentration of 1:1 and 2: 100 $\mu$ l.

### 6.5.2 Fabrication of PPCL mats.

The PPCL mats were synthesis as described above and the dried micobeads were dusted on to the mats. The PPCL was again electrospun over the micro beads to get a micro bead entrapped mats.

### 6.5.3 SEM observation

The Chitosan microbeads were < 70  $\mu$ m diameter and dispersed in the suspension. The beads dried on a wattmann filter paper were transferred directly to partially spun PPCL scaffold. The beads were entrapped by further electropinning of PPCL. The fiber entangled beads were clearly visualized without losing the morphology (Figure 9).

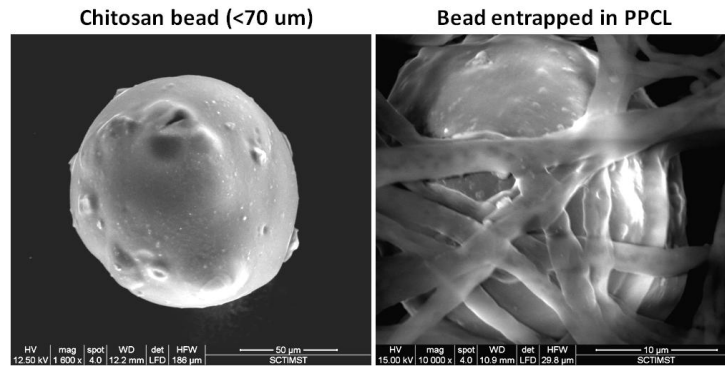


Figure 9 SEM image showing a single chitosan microbead loaded with antibiotic and microbead entrapped in PPCL fibers.

#### 6.5.4 Cytotoxicity analysis of the scaffolds.

General cytotoxicity of microbead entrapped scaffolds was carried out using L-929 cells by direct contact method (ISO10993-5). The scaffolds showed moderate toxicity.

#### 6.5.5 Haemocompatibility analysis

The hemocompatibility studies analyzed by measuring hemolysis and platelet adhesion using human blood showed the scaffolds to be hemocompatible. The average hemolytic value was 0.07 compared to normal hemolytic value (<0.1). The scaffolds showed  $32 \pm 12\%$  of platelet adhesion.

#### 6.5.6 Antimicrobial activity analysis:

Antimicrobial activity was determined by agar diffusion method on plates cultured with virulent strains of *S.aureus* and *E.coli*. No antimicrobial activity could be detected.

### 6.6 Cell culture with electrospun scaffold

#### 6.6.1 Cell culture on polymer scaffolds

Cell isolation was conducted after prior approval from the Institute Animal Ethics Committee [SCTIEC-20/jun/2012/177] of Sree Chitra Thirunal Institute of Medical Sciences and Technology. Primary keratinocytes were isolated from adult mice by double enzyme method (Dispase II and Zyme free solution). The keratinocytes were characterized by detecting the protein expression of cytokeratin 1 (CK-1) and cytokeratin 14 (CK-14) by flow cytometry. In normal skin CK-1 is expressed by cells on basal layer, CK14 by cells on suprabasal layer. The results showed positivity of CK-14 in the primary isolate.

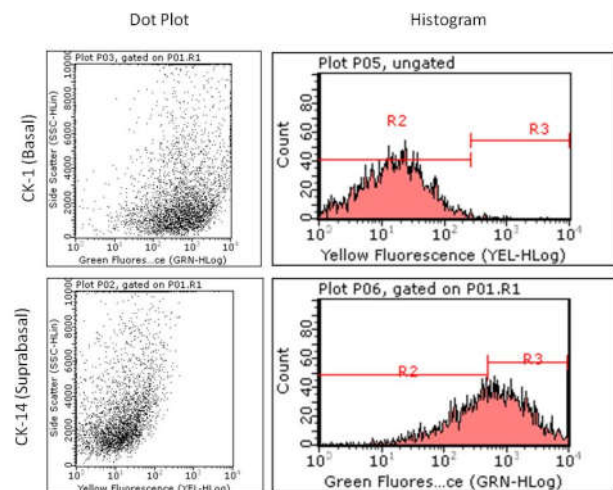


Figure 10 Flow cytometry of murine keratinocyte isolate showing negative for CK-1 and positive for CK-14.

#### 6.6.2 Construction of in vitro skin structure by monolayer transfer

The conventional approach of tissue engineering is limited by cell destructive enzymatic procedure and scaffold based techniques. These restrictions can be surmounted by a new method known as cell sheet engineering using thermosensitive polymer grafted surface that undergoes hydrophilic-hydrophobic transitions. The objective of the project was to develop of

cellularized biodegradable electrospun mats with at least one side completely covering with cells. Cell sheet technology offers this advantage and enables the lining of cell sheet construct on artificial scaffold.

The possibility of retrieving primary cells using thermoresponsive surface was initiated. The cell sheet technology requires thick cell monolayer or multilayer for efficient cell sheet transfer. Few patches of cultured primary cells could be retrieved upon lowering the temperature.

Primary keratinocyte culture was established on in house developed thermoresponsive (N-isopropylacrylamide – co – glycidyl methacrylate [NGMA]) polymer coated dishes. The cell patches were tried to transfer to fresh tissue culture dish by temperature variation with the help of electrospun CSPCL mats. The fibrous mats was kept over the cell patches and incubated for 24 h at 37 °C. The temperature was lowered and the electrospun mats was removed with cell patches attached on it. The cell patches were transferred to another fresh plate by incubation at 37°C (Figure 11).

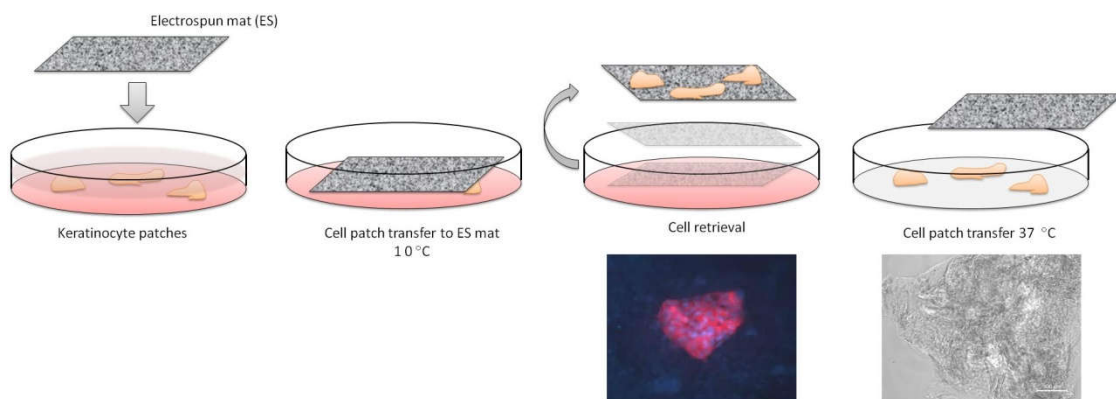


Figure 11 Illustration shows cell culture on thermoresponsive polymer and patch transfer using electrospun fibrous mats.

## 7. Salient Research Achievements

### 7.1 Summary of Progress:

#### 7.2 New Observations

- The in vitro cytocompatibility tests with L-929 fibroblast confirmed that CS-PCL is a good candidate for tissue engineering.
- Human keratinocytes (HaCaT cells) has been cultured on electrospun scaffolds and confirmed the cytocompatibility of CS-PCL scaffold for skin tissue engineering.
- Murine keratinocytes isolated with dual step method has been standardized. The isolated cells were positive for CK-14 which is the marker for cells of suprabasal origin.
- Various versions of PCL fibrous mats were modified to impart better degradation and cytocompatibility.
- Electrospun mats with porous PCL fibers have been evaluated for wound dressing application. To assimilate antimicrobial property to the wound dressing matrix, chitosan micro beads loaded with antibiotic has been sandwiched in PCL mats. Further analysis is ongoing.
- Preliminary experiments of transferring keratinocytes from thermoresponsive culture surface resulted in retrieval and transfer of small patches. Transfer of intact keratinocyte cell layer to electrospun mats has to be further optimized in order to satisfy large area tissue defects.

**7.3 Innovations**

- CS-PCL electrospun mat suitable for culture of skin keratinocytes.
- Electrospun mats with porous PCL fibers and Hydrolyzed porous fibers has been found to be a novel wound dressing material.

**7.4 Application Potential:****7.4.1 Long Term**

- Bioengineered skin substitute from stem cells.
- Preclinical in vivo efficacy studies of electrospun mats for wound regeneration.

**7.4.2 Immediate**

- A matrix for transfer of keratinocytes.
- An antibacterial matrix as wound dressing material.

**7.5 Any other**

- CS-PCL scaffold can also be used for fabricating vascular grafts.

**8. Research work which remains to be done under the project (for on-going projects)**

- Remaining objectives of 3<sup>rd</sup> year.
- Cell sheet transfer to electrospun mat.

<b>Ph.Ds Produced no</b>	<b>Technical Personnel trained</b>	<b>Research Publications arising out of the present project:</b>
None	1 JRF	1 Peer Reviewed Journal 2 under pipeline

**List of Publications from this Project (including title, author(s), journals & year(s))****A. Papers published only in cited Journals (SCI)**

1. Characterization and *In Vitro* Evaluation of Electrospun Chitosan/Poly( $\epsilon$ -Caprolactone) Blend Fibrous Mat for Skin Tissue Engineering- Tilak Prasad, Shabeena EA, Vinod D, Kumary TV and Anil Kumar PR – Journal of Materials Science Materials in Medicine 2014: *Accepted for Publication*.

**B. Papers published in Conference Proceedings, Popular Journals etc.**

1. Tilak Prasad, Vinod D, Kumary TV and Anil Kumar PR A Novel Strategy Of Gene Transfer To Cell Monolayer By Modulating Micro Environment, International Conference of Society for Education and Scientific Research on Biosciences: State - Of - The - Art Advancements, Kumarakom, Kottayam, Kerala, September 11-12, 2014.
2. Anil Kumar PR, New Concepts in Developing Biologically Relevant Scaffolds for Skin Tissue Engineering, International Conference of Society for Education and Scientific Research on Biosciences: State - Of - The - Art Advancements, Kumarakom, Kottayam, Kerala, September 11-12, 2014
3. Anil Kumar PR, Tilak Prasad, Shabeena EA, Vinod D and Kumary TV, In Vitro Evaluation of Chitosan Based Fibrous Mats Using Keratinocytes for Skin Tissue Engineering, International Conference on Medical Materials, Devices and Regenerative Medicine (MMDRM) 2014, 11-13 January 2014, Kathmandu, Nepal.

**C. Invited talks**

1. Anil Kumar PR, Advanced methods in developing bioartificial substitutes for tissue engineering, International Conference of Society for Education and Scientific Research on Biosciences: State - Of - The - Art Advancements, Kumarakom, Kottayam, Kerala, September 11-12, 2014.
2. Bioengineered Skin Construct by Cell Sheet Technology, First International and Third National Conference on Biotechnology, Bioinformatics and Bioengineering – Society of Applied Biotechnology, Tirupati, Andhra Pradesh, June 28-29, 2013.

**Patents filed/ to be filed:** 1 patent to be filed. There could be more products that can be decided while concluding the project.

1. Microbead loaded electrospun mats for wound care application.

Major Equipment (Model and Make)		Purchased in First year (2012-2013)			
Sl No	Sanctioned List	Procured (Yes/ No) Model & make	Cost (Rs in lakhs)	Working (Yes/ No)	Utilization Rate (%)
1	Syringe pump and accessories for electrospinning	Yes Holmarc	2,29,815	Yes	90%
2	Overhead stirrer	Yes Remi	9,080	Yes	70%