BIOFUNCTIONALIZED BIODEGRADABLE ELECTROSPUN MATRIX FOR WOUND HEALING APPLICATION

A dissertation submitted

by

SNEHA SUNDARAN. P

In partial fulfillment of the requirements
for the degree of

Master of Philosophy

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY
TRIVANDRUM – 695 011

DECLARATION

I, **Sneha Sundaran P**, hereby declare that I had personally carried out the work depicted in the dissertation entitled "**Biofunctionalized Biodegradable electrospun matrix for wound healing application**" under the direct supervision of **Dr. Anil Kumar. P.R**, Scientist D, Division of Tissue Culture, Department of Applied Biology, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India. External help sought are acknowledged.

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CERTIFICATE

This is to certify that the dissertation entitled "**Biofunctionalized Biodegradable electrospun matrix for wound healing application**" submitted by **Sneha Sundaran P** in partial fulfillment for the **Degree of Master of Philosophy** in Biomedical Technology to be awarded by this Institute. The entire work was done by her under my supervision and guidance at Division of Tissue Culture, Department of Applied Biology, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Trivandrum- 695012

Date : Dr. Anil Kumar. P.R

Place:

The dissertation

Entitled

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CONTENTS

ACKNOWLEDGEMENTS

Here I take the opportunity to express my gratitude to all those who have helped me in completion of my project and in presenting this dissertation in partial fulfillment of the Degree of Master of Philosophy.

I sincerely express my gratitude and respect to my guide Dr. P.R. Anil Kumar, Scientist-D, Division of Tissue Culture, Department of Applied Biology, Biomedical Technology Wing for his patience, inspiring guidance and scholarly supervision from the initial to the final level.

My deepest sense of gratitude to Dr. T.V.Kumary, Scientist in Charge, Division of Tissue Culture, Department of Applied Biology, Biomedical Technology Wing for her whole hearted support, advice and patience.

I am extremely grateful to The Director of SCTIMST, The Head, Deen, Biomedical Technology Wing for providing the necessary facilities and to complete the course successfully.

I am obliged to Dr. Jayabalan M, Scientist-G, Previous M.Phil course co-ordinator for his support and guidance. I am also thankful to Dr. Maya Nandakumar & Dr. Manoj Komath for their support and guidance as our course co-ordinators and Deputy Registrar for the academic support bestowed on us to enable us completing the M.Phil Programme.

With great pleasure I extend my heartfelt thanks to Mrs.Deepa K Raj, Mrs.Usha Vasudev, Dr. Bernadette K Madathil, Mr.Vinod D, Mrs Lakshmi.R.Nair, Mr Balu V Gopal and for their constant support and encouragement at all time.

I am also indebted to Dr. Lissy K Krishnan, Dr Anugya Bhatt and all Staffs at Thrombosis Research Unit for their timely help in allowing me to use their lab equipment which lead to the successful completion of my project.

My heartfelt thanks to Dr Shiny Velayudhan, Dr. Suresh Babu, Dr Roy Joseph, Dr. Ramesh.P, Mr. Nishad, Ms Jincy for arranging the facilities for Electrospinng, FTIR, Mechanical strength analysis, SEM analysis. I am extremely thankful to my batch mates Archana, Reesha, Riju, Sreelakshmi, Deepa, Jimna, Lekha, Prabha they were with me all the time, understanding, and supporting me whenever I feel discouraged and dejected. I express my deepest love and affection to my parents, my family members and my friends for their constant encouragement and moral support in my up's and down's.

LIST OF ABBREVIATIONS

PPCL	Porous Polycaprolactone- Fiber is porous
PPCL	Porous Polycaprolactone-Plane
PPCL-G	Porous Polycaprolactone-Grid
PLGA	poly(lactic-co-glycolic acid)
TBfPPCL-P	Thrombin Biofunctionalized PPCL- P
TBfPPCL-G	Thrombin Biofunctionalized PPCL- P
TCA	Trichloro Acetic Acid
NaOH	Sodium hydroxide
FT-IR	Furier-transforminfrared
MEM	Minimal essential medium
FBS	Fetal bovine serum
FDA	Fluorescein diacetate
PI	Propidium Iodide
CO2	Carbon dioxide
FITC	Fluorescein isothiocynate
PBS	Phosphate buffered saline
EDTA	Ethylene diamine tetra acetic acid
BSA	Bovine serum albumin
SEM	Scanning electron microscopy
E-SEM	Environmental SEM
F-ESEM	Field emission SEM
SWF	Simulated Body Fluid
rpm	Revolution per minute.
NaCl	Sodium Chloride
Cacl2	Calcium Chloride
SAP	Super absorbent polymer
THF	Tetrahydrofuran
DMSO	Dimethyl sulfoxide
FITC	Fluorescein isothiocyanate

LIST OF NOTATIONS

h	Hour
min	Minute
sec	Seconds
cm	Centimeter
cm^2	Centimeter square
M	Molar
mg	Milligram
g	Gram
µg	Microgram
µL	Microlitre
µm	Micrometre
mL	Milliliter
^oC	Degree Celsius
%	Percentage

LIST OF FIGURES

SYNOPSIS

Skin is the largest organ in the body with three main functions protection, regulation and sensation. Wounding affects all these functions. A skin wound may be defined as a break in the epithelial integrity of the skin and affect all normal functioning. External wound dressing materials are important to protect and restore the functions of skin. Wound dressings are the most commonly used therapeutic agents, for wound healing. For years, man has used different materials such as clay, linen, honey, animal fats, and vegetables fibers for wound dressing. Continuous developments have led to extensive use of new bandages with improved performance. In 19^{th} century various textile fibers such as cotton, silk and wool with varying structure such as knitted, nonwoven and composites start to used for wound management. Today's wound dressing materials are usually based on synthetic polymers.

The thesis consist of 4 chapters namely Introduction, Materials &Methods, Results and discussion, Summary and conclusion. Chapter 1 includes the background of work, review of literature in relevance to the study.

Aim of this study

- Fabrication of electrospun mat and its characterization.
- Biofunctionalization of electrospun mat with blood coagulating factor, thrombin, and its efficiency analysis
- Evaluation of cytotoxicity and cytocompatibility of biofunctionalized electrospun mats.
- Fabrication of biofunctionalized and Biodegradable electrospun mat for large area tissue defect.
- Device models of wound dressing with super absorbent property for exuding wounds.

Chapter 2 describes the Materials and Methods adopted in this study to achieve the objectives. Porous Polycaprolactone (PPCL) mat was synthesized by electrospinning method and its physicochemical property was analyzed through SEM, FTIR and mechanical strength analysis. Biocompatibility was analyzed through cytotoxicity, cytocompatibility and hemocompatibility tests. PPCL mat was biofunctionalized with

thrombin to achieve hemostatic property of wound dressings and its biocompatibility test was done. Efficiency of biofunctionalization was analyzed through protein estimation and SDS-PAGE analysis. An *ex vivo* wound model was proposed to evaluate the hemostatic efficiency of Thrombin Biofunctionalized PPCL mat when applied on a bleeding wound. To increase the biodegradation of PPCL wound dressings PPCL-PLGA (poly (lactic-co-glycolic acid) electrospun mat was synthesis and conduct its degradation study. A device model was proposed by using superabsorbent polymer along with PPCL mat for highly exuding wounds.

Chapter 3 and 4 contain the results of various experiments conducted and the relevant discussion. Physicochemical and biological evaluation of PPCL mat showed that it is suitable for wound healing applications. Protein estimation and SDS-PAGE data confirm the thrombin biofunctionalization and its biocompatibility analysis revealed that biofunctionalization was also compatible for skin wound applications. Presence of fibrin protein as an evidence of biofunctionalization was detected from samples in *ex vivo* wound model. Biodegradation study of PPCL-PLGA (poly (lactic-co-glycolic acid) electrospun mat data gave explanation that it is biodegradable. Experiments conducted using device model proposed that it is suitable for exudates absorption. Chapter 5 is the summary and conclusion of the work followed by bibliography.

Chapter 1

INTRODUCTION

1.1 Background

1.1.1 Structure and functions of skin

Human skin is the largest organ in the body with 15%of the total body weight and cover approximately $2m^2$ area. It receives one third of the body's circulating blood volume and have varies from 0.5mm to 6 mm. Skin has mainly 3 functions protection, regulation and sensation. The human skin can be divided as outer epidermal layer, middle dermal layer and inner hypodermal layer. Avascular Epidermal layer is derived from embryonic ectoderm and act as barrier and protect the body from infection. Hair nails and sweat glands are the derivatives of skin. Due to the lack of blood vessels epidermis receives oxygen and nutrients from the dermal capillary through diffusion. Skin is a dynamic organ in a constant state of change, as cells of the outer layers are continuously shed and replaced by inner cells moving up to the surface. Epidermis is a stratified squamous epithelium consists of keratinocytes and dendritic cells. Melanocytes, Langerhans cells and merkal cells are the epidermal non keratinocyte cell populations. Epidermis is divided into four separate layers on the bases of distribution and morphology of keratinocytes which moving from lower layer to surface. These layers are Stratum basale, Stratum spinosum, Stratum granulosum, Stratum lucidum, Stratum corneum, Porous basement membrane between the dermis and epidermis allow the exchange of cells and fluid and hold the two layers together. The toughest layer of the skin epidermis is also known as corium and is derived from embryonic mesoderm. It protects the body from stress and strain due to the presence of connective tissue. Dermis is found below epidermis and is composed of tough extracellular matrix (ECM). Dermis is populated with fibroblast cells and is vascularised and innervated. Network of blood vessels functions to provide nutritional support, immune surveillance, wound healing, thermal regulation, hemostasis and inflammatory response. The dermis is composed of upper thin papillary layer and lower reticular layer. Inner hypodermal layer is made up of fatty

subcutaneous tissue and consist of large blood vessels and nerves, it insulates the body and absorbs shock. [1, [2]

1.1.2 Wound and Wound Classification

A wound may be de fined as a break in the epithelial integrity of the skin or may also be defined as a loss or breaking of cellular and anatomic or functional continuity of living tissue. According to the Wound Healing Society, wounds are physical injuries that result in an opening or break of the skin that cause disturbance in the normal skin anatomy and function [3]. Wound results in the loss of continuity of epithelium with or without the loss of underlying connective tissue [4]. Based on the length of the time to heal wounds are categorized as acute and chronic wounds. Acute wound is an injury to the skin that occur suddenly rather than over time. These types of wounds are caused by trauma; mechanical damage induced by sheer and stabbing action of hard objects; exposure to extreme heat, irradiation, electrical shock, and/or irritated with corrosive chemicals, usually healable within 8 to 12 weeks. Acute wounds normally proceed through an orderly and timely manner that results in sustained restoration of anatomic and functional integrity. A chronic wound is a wound that does not heal in an orderly set of stages and within predictable time period to establish a sustained anatomic and functional result. These type of wounds result from diseases like diabetes, tumors and severe physiological contaminations, usually chronic wounds take more than 12 weeks to heal [5, [6, [7, [8]. As per depth wounds are classified into superficial wounds, Partial-thickness wounds, full-thickness wounds and Deep wounds. Deep wounds are complicated wounds formed by laceration of blood vessels and nerves, wounds penetrating into natural cavities; Full-thickness wounds are damages extending to skin and the subcutaneous tissue and the skin edges are spaced out. Partial-thickness wounds, involve skin loss up to the lower dermis. These wounds normally take 10-21 days to heal with combination of re-epithelization, wound contraction and scar formation. Superficial wounds occurs at the epidermis and the dermis up to the dermal papillae and are occur from cuts, skin tears, abrasions, punctures and burns [9]. A burn may be superficial involving only epidermal layer of the skin [10]. These types of wounds heal within 10 days in the absence of infection and appropriately maintained wound environment. They heal by epithelization from surviving pilosebaceous units such as sebaceous glands, sweat glands and hair follicles without scar formation and wound contraction [11]

1.1.3 Wound Healing

Physiological system starts to repair damaged tissue after an injury to make it normal through a series of time dependent events known as wound healing. These physiological events are Hemostasis, Inflammation, Proliferation, and Remodeling. For a wound to heal successfully, all four phases must occur in the proper sequence and time frame.

Hemostasis: Hemostasis can be defined as a tightly regulated process that maintains the blood flow through the vasculature simultaneously as a thrombotic response to tissue damage occurs, usually completed within hours. Hemostasis is achieved by 3 mechanisms such as vasoconstriction, Platelet plug formation and Coagulation [12]. Interaction of fibrinolytic system is also required for successful completion of hemostasis [13]

Vasoconstriction: Constriction of blood vessels start immediately after injury to reduce blood flow through blood vessels, as a result minimal blood loss occur at the site of injury compared to the blood loss that would have occurred without vasoconstriction. Damaged blood vessels expose the subendothelial collagen to platelets which trigger platelet adhesion and activation. Platelet Adhesion is accelerated by VonWillebrand factor. The activated platelets secrete serotonin and other vasoconstrictor which cause constriction of the blood vessels. [14]

Platelet plug formation: Activated platelets at the site of injury also secrete adenosine diphosphate (ADP) and thromboxane A2 these attract and activate more platelets in the presence of platelet activating factor (PAF). These aggregated platelets together form a loose temporary platelet plug or hemostatic plug, which closes the ruptured vessel and prevents further blood loss [14].

Coagulation: Blood coagulation is the transformation of liquid blood into a semisolid gel form due to the conversion of blood protein fibrinogen into insoluble polymeric net work of fibrin [15]. This fibrin network stabilizes the temporary platelet plug and completely seals the injured site to prevent blood loss. A dozen of protein activate and participates in a sequential manner to achieve blood coagulation is known as

coagulation cascade/clotting cascade [16]. Three stages of clotting process are the formation of prothrombinase, conversion of prothrombin to thrombin and conversion of fibrinogen to fibrin. In the presence of FIV(Ca^{2+}) and FXa together with FVa form a complex is known as prothrombinase complex [17]. Prothrombinase complex can form by two pathways extrinsic Pathway and Intrinsic pathway. Intrinsic pathway is initiated by blood making contact with a foreign body and damaged endothelium of vessel wall [16]. Collagen beneath the endothelium is exposed. When factor FXII (Hageman factor) come in contact with collagen it is converted into FXIIa in the presence of the presence of kallikrein and high molecular weight kinogen. FXIIa converts FXI (Plasma thromboplastin antecedent) into FXIa in the presence of high molecular weight kininogen. In the presence of Ca^{2+} XIa activate FIX(Antihaemophilic factor/Christmas factor) into FIXa. FIXa activate FX (Stuart –Prower factor) as FXa in the presence of FVIII and Ca^{2+}. When platelet comes in contact with collagen of damaged blood vessels it gets activated and release phospholipids. Now the activated FX reacts with platelet phospholipids and factor FV(Proacclerin/Labile factor) to form prothrombinase complex in the presence of calcium ions [14].

Extrinsic pathway is quicker and has fewer steps than intrinsic pathway.[17] This pathway is initiated by contact of blood with FIII (tissue factor/thromboplastin) which is released by injured tissue. FIII is a proteolytic enzyme which binds and activates FVII to FVIIa.Now FX is activated to FXa by FIIIa- FVIIa complex. Activated FX reacts with factor FV and phospholipids component of tissue factor to form prothrombinase complex. This reaction requires the presence of Ca^{2} [14]. After blood vessel rapture clotting occurs by both pathways simultaneously. Tissue factor initiates the extrinsic pathway, whereas contact of FXII and platelets with collagen in the vascular wall initiates the intrinsic pathway [14].

Thrombin is the key enzyme in coagulation pathway .In the presence of Ca^{2+} prothrombinase complex synthesized from extrinsic and intrinsic pathway convert prothrombin into thrombin. Once thrombin is formed it initiates the formation of more thrombin and activation of more FV to FVa. FVa accelerate prothrombinase production which also convert prothrombin(factor II) into thrombin [14].

Fibrinogen (Factor I) is activated and form stable fibrin threads in the presence of thrombin, activated FXIII (Fibrin stabilizing factor) and $Ca^{2+.}$ At last this fibrin threads are aggregate with other blood cells to form mesh work of stable clot. After about 30 to 45

minutes, the serum oozes out of the clot. The process involving the contraction of blood clot and oozing of serum is called clot retraction and after clot retraction fibrinolysis is activated by plasmin [14].

Inflammation: Once the bleeding is controlled white blood cells, body's "soldiers" are recruited to clean up wound bed along with breakdown of any devitalized tissue and elimination of excess bacteria. Success inflammatory phase allow wound to enter into rebuilding by producing growth factor for proliferation rather than inflammation. In case of acute wound healing inflammatory phase lasts up to 3 days. However wound complicated by necrosis or infection the inflammatory phase is prolonged and wound healing is delayed. Delayed inflammation promotes scar tissue formation. Diabetes is the clinical conditions associated with prolonged inflammation. Ischemia /hypoxia also produce prolonged inflammation.

Proliferation and Repair: During this phase wound surface is covered with new epithelium that restores the bacterial barrier and vascular integrity. Proliferative phase include epithelization, neoangiogenesis and matrix deposition/collagen synthesis. This stage occurs 1-3 weeks after injury.

Remodeling: Collagen synthesized in proliferative phase is characterized by poorly organized fibers having limited tensile strength and gradually leads to scar formation. Newly formed Extracellular matrix (ECM) with scar is highly stiff in nature because it lacks elastin which provides the elasticity of normal uninjured skin. Remodeling phase involve synthesis of new collagen for normal ECM and degradation of scar, and is regulated by fibroblasts and ECM protein. Remodeling begins 3 weeks after injury and take month to several years to achieve physiologic completion [2].

1.1.4 Wound management

The management of wound relates to conditions that prevent aggravation of wound and maintain a conductive environment for wound healing. The main objective of wound management is to provide effective healing of the wound within minimum time and without causing further trauma. Selection of appropriate wound dressing material is important for each type of wounds for quick healing.

1.1.5 Wound dressings

Wound dressings are the most commonly used therapeutic agents, for wound healing. For years, man has used different materials such as clay, linen, honey, animal fats, and vegetables fibers for wound dressing. Continuous developments have led to extensive use of new bandages with improved performance. In 19^{th} century various textile fibers such as cotton, silk and wool with varying structure such as knitted, nonwoven and composites start to use for wound management. Today's wound dressing materials are usually based on synthetic polymers [8]

1.1.6 Properties of ideal wound dressing

A wound dressing materials should have various quality to be ideal such as

- It should remove excessive exudates from the wound but not allow the wound to dry out and have to maintain moist environment.
- It should allow gaseous exchange so that oxygen, carbon dioxide and water vapor can pass into and out of the dressing.
- It should provide thermal insulation and maintain wound temperature at 37℃
- It should be impermeable to microorganisms and minimize contamination from outside the wound.
- It should be free from toxic contaminations.
- It should be non traumatic and not adhere to the wound, so that no damage is done to granulating tissue on removal.

In addition to above characters following properties are also important to consider while choosing a wound dressing material, include material should be provide proper environment for healing, user friendly, easy for application and removal, simply the treatment, cost effective, compatible with the wound, minimize need for secondary dressings, remain in place [18, [19].

1.2 Review of Literature

The skin has been described as largest organ in the body. Their functions are numerous and any injury to its various layer increase the vulnerability of the organism to additional biological and physical hazards resulting in wound. Wound care is an essential aspect of human survival. Wound care is "the provision of the appropriate environment for healing by both direct and indirect methods together with the prevention of skin breakdown". A wound is defined as a disruption in the continuity of the epithelial lining of the skin or mucosa resulting from physical or thermal damage [20].

1.2.1 Classification of Wounds

1.2.1.1 *Based on the thickness*

Based on the thickness wounds are classified into superficial wounds, involving only the epidermis and the dermis up to the dermal papillae. Partial-thickness wounds, involving skin loss up to the lower dermis (Part of the skin remains and shafts of hair follicles and sweat glands are left over.) Full-thickness wounds, involving the skin and the subcutaneous tissue (Tissue loss occurs, and the skin edges are spaced out). Deep wounds, including complicated wounds (eg, with laceration of blood vessels and nerves), wounds penetrating into natural cavities, and wounds penetrating into an organ or tissue. Based on the severity burn wounds are coming under either of these four [9].

1.2.1.2 *Based on Morphology*

Based on morphology wound are classified into closed and open wounds closed wounds includes contusion, hematoma and abrasion. Contusion-type injuries involve damage to soft tissues, small blood vessels and deep tissue layers, resulting in their separation, but the anatomy of the skin remains intact. Edema, and in later periods, atrophy and defective pigmentation are observed in wound and the healing is delayed. Vessel rupture or hyperaemia due to vessel damage is called hematoma and wounds such as scrapes are termed abrasions. The healing process is very painful because this type of wound involves damage to sensory nerves and the wound can easily become infected. Open wounds includes lacerations, cutting-pricking tool wounds, gunshot wounds, surgical wounds, insect bites and stings, radionecrosis, vascular neurological and metabolic wounds. Wounds except for lacerations cause serious damage to tissues beneath

the skin. In laceration type wounds, skin and subcutaneous tissue have been destroyed, but deep tissues remain healthy. The anatomical integrity of tissues is damaged in cutting pricking tool wounds without any tissue damage at the edges of the wound [21]

1.2.1.3 *Based on the healing time*

Based on the healing time wounds are classified into acute and chronic wounds. Acute wounds include surgical wounds, traumatic wounds and burns. Chronic wounds include pressure ulcers, Leg ulcers, and diabetic foot ulcers. The differences between these two are the time taken for wound to heal. Acute wounds heal quickly and chronic wounds take much longer to heal. Some time acute wound become chronic. In chronic wounds the usual orderly process of healing that occur in acute wounds-hemostasis, inflammation, proliferation and remodeling is disrupted at one or more points in the process resulting in delayed healing [22].

1.2.2 Wound healing products

1.2.2.1 *Gauze*

The use of gauze to dress and bandage wounds has its origins in ancient times, having been firmly established by the fifth century BCE and is still in use today. Gauze represents woven and non-woven bandages. Woven gauze is 100% natural cotton and nonwoven gauze consists of synthetic dressing materials. Woven gauze is problematic in dressing and packing wounds as it sheds fibers when cut and may leave debris in the wound bed when removed. It is also absorbent and tends to stick to the wound, resulting in trauma upon removal as it quickly dries out the wound, becoming trapped [23, [24]. Today, woven gauze is seen as a 'wet to dry' dressing and utilized in a range of wound care strategies. Despite its non-selective mode of physical debridement, trauma to the wound bed and resultant pain, it is still the most utilized wound dressing in the world. It is also utilized as a vehicle for antimicrobial agents eg: Kerlix [25].

1.2.2.2 *Film Dressings*

These dressings can be used as primary or secondary dressings. It is usually not covered by a secondary layer. Film dressings can also be used as a barrier to protect an area of wound and often form part of the construction of other dressing such as hydrocolloids, foam, hydrogel sheets and composite dressing, which are made up of

several material with the film being used as the outer layer. Film dressing have long history in wound management and are frequently used in day-to-day clinical [26]. The film dressing allows water vapor permission, thus reducing fluid loss from the wound site as well as it is also occlusive to bacteria, thus preventing secondary infection of the wound. The dressing is usually left on wound bed for several days depending on the amount of fluid produced by the wound. This type of wound dressing is that the wound may be visualized without the need to remove the dressing eg: BAND-AID® [27].

1.2.2.3 *Hydrocolloids*

Hydrocolloid dressings are composed mainly of cellulose and it is occlusive nature thus it provides moist environment to stimulate new tissue formation which helps to optimal healing of various type of wound. E.g. pressure ulcers, leg ulcers, surgical wounds, abrasions and minor burns [28]. Hydrocolloids have an outer waterproof layer that is impermeable to bacteria also provide thermal insulation enabling the wound bed to remain near body temperature. The inner hydrocolloid layer interacts with wound exudate to form a gel which promotes autolysis. Hydrocolloid dressings are much more complicated than hydrogels because they contain a variety of constituents, such as methylcellulose, pectin, gelatin, and poly isobutylene [29]. some of them also contain alginate. After contact with the wound surface, hydrocolloids slowly absorb fluids, leading to a change in the physical state of the dressing and to the formation of gel covering the wound. Thus, they are called interactive dressings. Depending on the choice of product, hydrocolloids are suitable for the dressing of both acute wounds and chronic wounds. Initially, hydrocolloid wound dressings need to be changed daily (depending of the exudate level), but, once the exudate has diminished, dressings may be left on the wound surface for up to 7 days. With a few exceptions, hydrocolloids require a secondary dressing to be fixed in place. Hydrocolloids should not be used on infected wounds Eg: Duoderm® Cgf®, Medline Exuderm® [30].

1.2.2.4 *Alginate dressing*

Alginate product derived from the seawood with differing ratios of D-Mannuronic and L-Guluronic acid of the alginate and the balance of sodium and calcium alginate within the dressing. Alginates rich in D-Mannuronic form soft amorphous gels that disperse more in solution. Alginates rich in L-Guluronic acid tend to swell more in solution, whilst retaining their basic structure. On contacting blood, the calcium ions in the

alginate are exchanged for sodium ions in the blood, increasing the solubility of the dressing i.e. gel-forming [31]. Replacement of some of the calcium ions by sodium ions in the dressing has been proposed to accelerate gel formation but may reduce its ability to activate the clotting cascade eg: Maxorb® Extra [32].

1.2.2.5 *Hydrogel*

Hydrogels are hydrophilic natured three-dimensional networks, held together by chemical or physical bonds. Hydrophilic groups such as hydroxyl (OH) and carboxyl (COOH) on the polymer chains absorb and store water. If enough interstitial space exists within the network, water molecules can become trapped and immobilized, filling the available free volume. This is the quality that brings about the specific benefit of hydrogels in wound treatment [33]. They immediately function as moist wound dressings. At the same time they are capable of absorbing contaminated exudates and safely retaining them within the gel structure. The absorption of secretions causes an expansion of the cross-links in the polymer chains. The basic physical features of hydrogel dressings can be specifically modified, according to the properties of the polymers used [34] . Hydrogels save the wound from fluid loss, are capable of providing the lesion with additional moisture, and securely protect it against external noxae. Under the dressing a micro climate is developed, that stimulates and regulates all cellular activities and nutritional processes during the individual phases of wound healing. Additional advantages such as transparency, cushioning effect, cooling effects etc. considerably increase the utility value of hydrogels, in particular concerning patient comfort and ease of application. The transparent hydrogels have the advantage in that the wound healing process can be easily monitored without removing the dressing [35]. The high moisture content and the softelastic, cushioning properties of the hydrogel almost act like a “second skin”. The hydrogel dressing removal is almost painless because hydrogel does not stick to the wound but it remains intact in the wound site due to the presence of hydrophilic groups, which forms secondary bonds with the hydrophilic groups at the wound surface. Hydrogel stays permanently moist and can even after prolonged application be removed without pain and risk of wound irritation. Moreover, malodour is reduced with hydrogel dressings, because the odour molecules are retained in the gel structure along with the absorption of secretions. The treatment of wounds with hydrogels may bring about great relief for both the patient and the nursing staff. The acceptance of a wound therapy with

hydrogel is thus, in general, very high on the part of the patient Aquacel® Hydrofiber® [36].

1.2.2.6 *Foam dressings*

Hydro cellular foam dressing's use on moderate to highly exuding wounds. The dressings absorb, retain and transpire to achieve the optimal balance of fluid .This process helps to promote faster healing by maintaining an optimal wound healing environment and reduce the risk of maceration by not allowing the wound to become too wet Adhesive foam dressings are also used as secondary dressings to help primary dressings remain in the wound bed while also assisting with absorbency. Foam dressings are not associated with debriding wounds [37] eg: Optifoam®'.

1.2.2.7 *Silicone dressings*

Silicone is used either as a contact dressing or as the contact layer within a dressing, for example, Mepilex, a polyurethane foam membrane coated with a soft silicone layer [38]. It is also used as a coating on materials like non-woven polyester nets. In negative pressure therapy, silicone-coated polyester enhances healing rates in sheep models and may assist in the prevention of hypertrophic scarring when combined with pressure therapy [39]. Dressings incorporating soft silicone contact layers adhere to dry skin while remaining nonadherent to the wound site, resulting in atraumatic removal and a decreased risk of damage to the wound site upon dressing changes [40]. Silicone can be used on a range of acute and chronic wounds as it is incorporated in many different bandaging strategies eg:Mepitel, Mepilex Ag.

1.2.2.8 *Antimicrobial dressings*

Honey dressings(eg:Derma Sciences MediHone), Iodine dressings(Inadine dressings), Silver dressings(Sorbsan Silver), Antibiotics incorporatd dressings are some of the antibacterial dressing used for infected wounds and also to wound infections [41]

1.2.3 Types of wound dressings

1.2.3.1 *Passive Dressings*

These are traditional dressing materials that embrace gauze, tulle and lint. They fulfill very few properties of ideal dressing materials. Most of them used as secondary

wound dressings because of intense adhesion while drying the wound bed, which leads to further trauma. Gauze is manufactured as bandages, sponges, tubular bandages, and stocking. These dressings can stick to the wounds and disrupt the wound bed when removed. Therefore, these are suitable for minor wound, e.g. Multisorb. Greasy gauzes consisting of Tulle gauze and petroleum jelly. This dressing does not stick to the wound surface and is suitable for a flat and shallow wound with minimal to moderate exudates, e.g. Jelonet

1.2.3.2 *Interactive dressings*

Interactive dressing materials consist of polymeric films and foams which allow transport of oxygen and water vapor, prevent microbial attack and are transparent in nature. These films are suitable for wounds with low exudates and have property to stimulate wound healing cascade. Polymeric films are transparent and also suitable for shallow wound with low exudates eg: Tegaderm. Foams are designed to absorb large amounts of exudates. They are not used for low exuding wounds as they will cause dryness and scabbing, e.g. Allevyn. Amorphous gels are not cross-linked. They are used for necrotic or sloughy wound beds to rehydrate and remove dead tissue. They are not used for moderate to heavily exudating wounds, e.g. Intrasite.

1.2.3.3 *Bioactive dressings*

Bioactive materials are also known as active wound dressing materials (AWD). These are capable to deliver substances active in wound healing either by delivery of bioactive compounds or dressings are constructed from materials having endogenous activity these include hydrocolloids, alginates, collagens and chitosan. Hydrocolloids are semi-permeable polyurethane film in the form of solid wafers; contain hydroactive particles as sodium carboxymethyl cellulose that swells with exudates or forms a gel. Depending on the hydrocolloid dressing chosen they can be used in wounds with light to heavy exudates, sloughing, or granulating wounds. For example DouDERM [8] Seaweed derived alginates are used to synthesize soft and non woven calcium-sodium alginate dressings, usually used for wounds having high drainage of exudates and are capable of absorbing 20 times their weight. These are usually non adhesive and form gel in contact with fluid but seem to be adhesive and not form gel in no exudates wounds. These types of dressing are available in the form of sheets and ropes. e.g. Kaltostat [42]. Collagen is a natural scaffold in tissue and is the major component of the extracellular matrix. Collagens

dressings are available in pads, gels or particles. They promote deposition of newly formed collagen in wound bed, absorb exudates, provide a moist environment and promote wound healing cascade [43]. Hydrofibers are soft nonwoven pad or ribbon dressing made from sodium carboxymethyl cellulose fibers. They draw and lock the fluid away from the wound bed and become gel contact with fluid. Fibers in this types of dressings provide adhesion between wound and dressings without invading tissue growth to the dressing materials, that is beneficial for easy application, maintenance and removal of materials e.g. Aquacel [44].

1.2.4 Polymeric non-woven fibers as wound dressing

These are interactive type of wound dressing materials. Nonwoven are the materials of alternative for several wound care, surgical and surgery dressing applications owing to recent formulation and producing advancement and materials properties like absorbent, bacterial barrier, soft, stretchy, and liquid repellent. One of the techniques used to produce non-woven fiber is electrospinning. Technique of electrospinning was invented by Formhals in 1934. Electrospinning is type of fiber fabrication method that employs the use of an electrical charge to product micro and nano-scaled fibers from a liquid. The experimental setup consist of polymer solution is loaded into a syringe and forced to the needle tip by a syringe pump. A positively charged electrode is attached to the needle and when voltage is applied, a static electric field is created causing the droplet of polymer at the end of the syringe to be stretched into a conical shape known as the Taylor cone. With increasing electrical field, the discharged polymer solution jet is elongated and whipped continuously by electrostatic repulsion causing the solvent to evaporate. The charged polymer fiber is deposited on a grounded collector. This technique produces fibers with high surface area. The electrospinning technique produces continuous polymeric nanofibers and allows the control of surface area, fiber diameter and fiber density [45]. Electrospun mats have been used for cell cultivation, wound dressings, drug delivery and enzyme immobilization [46, [47, [48, [49]. Wound dressings synthesized by electrospinning have some special properties. Fibers of nano to micro structure with their pores and large surface area provide good hemostatic activity even without any hemostatic agents. Due to the high surface area to volume ratio of fibers they exhibit high water absorption rate use of hydrophilic polymer increase the water absorption even more. its semipermiable nature due to the presence of small pore size allow gaseous exchange, prevent microbial invasion, provide appropriate moisture

environment and allow respiration of the cells which prevent the wound to dry up. Ultra fine structure of the nonwoven fine fibers provide excellent conformability, Conformability or ability to confirm to the contour of the wound is considered as the parameter to assess the flexibility and resiliency of the medical dressings which provide better coverage and protection of wounds from infection. Electrospun fibers along with antiseptics, antifungal agents, vasodilators, growth factors and even cells can synthesize biofunctionalized wound dressings.

Polymers such as cellulose, alginate, dextran, chitosan, hyaluronic acid,poly-β-hydroxy butyrate, poly- β-hydroxy valerate, collagen, fibrin, poly-L-lactic acid, poly glycolic acid, PLGA, poly-β-malic acid (PMLA), Polycaprolactone (PCL) , Poly-L-lysine citramide (PLCA). Poly-L-lysine citramide- imide (PLCAI), Poly amino serinate (PAS), Polydioxanone (PDS) etc...are some of the polymers present in commercially available nonwoven electrospun wound dressing materials [8].

1.2.4.1 *PCL as a wound dressing materials*

Electrospun PCL is widely used for tissue regeneration and wound healing application due to its high biocompatibility and the similarity of its nanofibers to collagen fibrils of natural extracellular matrix [50]. Sustained release of the biomolecule is possible because of large surface area of fibers present in mat which allow strong interaction with tissue [51]. PCL Silver-loaded zirconium phosphate nanoparticles encapsulated PCL electrospun fibers is a materials for potential wound dressings applications because of its bacteriostatic nature and biocompatibility [52].

1.2.5 Hemostatic agents

Topical hemostatic agents fall into following categories based on their primary components

1.2.5.1 *Mechanical hemostats*

Mechanical hemostatic agents are available in the form of absorbable sponge, foams, pads, or other materials with topical hemostatic agents. Porcine gelatin (Gelfoam®, Gelfoam® Plus, Surgifoam®), cellulose (Surgicel®, Surgicel Nu-Knit®), bovine collagen (Avitene® sheets, UltrafoamTM collagen sponges), and polysaccharide spheres (Arista®) are the commercially available mechanical hemostasis and are most

useful in situation of minimal bleeding. These activate the extrinsic coagulation pathway and form fibrin clot at the bleeding site. So these agents are not applicable in patients with impaired coagulation cascade. Mechanical agents with thrombin have an intrinsic clotting efficiency.

1.2.5.2 *Active hemostats*

Topical thrombin that stimulates fibrinogen at the bleeding site to produce a fibrin clot is known as active hemostats. These agents are applicable in patients with both intact and impaired coagulation cascade. Commercially available active hemostatic gents are bovine thrombin (Thrombin-JMI®), recombinant thrombin (Recothrom®) and pooled human plasma thrombin (Evithrom®) and are available in powdered form. That is easily reconstituted with saline when needed for use. These are more effective than mechanical hemostasis for controlling local bleeding.

1.2.5.3 *Flowable hemostats*

Flowable hemostatic agents have the properties of both mechanical and active hemostats.eg, Surgiflo® & FloSeal®. Surgiflo is a porcine gelatin that is used in combination with bovine or other thrombin and FloSeal includes bovine gelatin micro granules combined with human pooled plasma thrombin and calcium chloride, these require two to three minutes of preparation time and are applied using a syringe. These agents are also ideal for treating patients who may have a small entry wound leading into a larger cavity, such as a gunshot wound.

1.2.5.4 *Fibrin sealants*

Fibrin sealants are absorbable dressings containing high concentration of both fibrinogen and thrombin. These agents are appropriate for patients who do not have sufficient fibrinogen to form clot. Eg: Tisseel® and Evicel®, both consist of human thrombin and fibrinogen.

1.2.5.5 *New Dressings*

New dressings include hemostatic bandages like HemCon® and QuikClot®. HemCon is made up of chitosan. Mucoadhesive property of Chitosan enable the bandage to stick to the wound, they also attack red blood to create a seal over the wound.

Nonwoven gauze with kaolin is the composition of QuikClot, which Initiates clotting by activating factor XII and attract red blood cells at the inured site [53]

1.2.6 Super absorbent polymer as wound dressing materials.

A polymer able to absorbe 100 times its original volume is considered as super absorbent polymers. Two classes of absorbent polymers are non-ionic and ionic polymers. Examples for nonionic super absorbent polymers are Polyacrylamides, polyethylene oxide, polyvinyl alcohol and gelatin. Polyacrylic acid is an ionic super absorbent polymer. The difference between traditional absorbent materials and superabsorbent material is their mechanism of absorption. Traditional materials use convective mechanism but superabsorbent materials use diffusive mechanisms for fluid absorption. Due to the cross links superabsorbents hold the particle together and not dissolved in the water. Presence of ions increases the absorption capability of polymers due to the strong ionic-dipole interactions [54]. Exudate control is crucial in management of chronic wounds. Most Super absorbent polymers (SAP) are cross-linked networks of flexible polymer chains with abundant ionizable carboxyl groups, typically joined with sodium ions. Fluid absorption occurs through osmotic pressure caused by the concentration gradient of electrolytes inside and outside the SAP particles. This process is accompanied by the detachment of the sodium ion, which leaves the charged carboxyl group behind, and the unfolding of the macromolecule chains, due to the repulsion forces of the negatively charged sites. This allows the SAP to swell and absorb large amounts of liquid. During this process, SAPs also take up and retain proteins, cell debris and even micro-organisms. *In vitro* studies shows that super absorbent polymers have capability to reduce concentration of Matrix metalloproteinase and polymorphonuclear elastase which are responsible for high rate of inflammation [54]. XTRASORB is a Sodium Polyacrylate containing flexible, soft superabsorbent dressings designed to manage moderately to heavily exudating wounds.

1.2.7 PLGA (poly (lactic-co-glycolic acid) as wound dressing materials.

PLGA is a copolymer of polylactic acid and glycolic acid and FDA approved therapeutic devices due to its biodegradability and biocompatibility. In the last years, PLGA has been used for medical applications, generating tremendous interest in this field due to its excellent biodegradability and biocompatibility they are natural polymers or synthetic in origin. The PLGA polymer is biodegraded into glycolic and lactic acid.

Glycolic acid enters the tricarboxylic acid cycle or it is excreted in the kidney and eventually eliminated as water and carbon dioxide. Lactic acid enters the tricarboxylic acid cycle and is metabolized, further it is eliminated from the body as carbon dioxide and water [55]. One of the simple method to faster wound healing is the application of exogenous lactate that accelerate angiogenesis [56]. PLGA blend of synthetic polymers is used in electrospinning to produce new scaffold materials. By mixing PLGA with another polymer material, the physical properties of PLGA, such as hydrophobicity, degradation rate, shrinkage behavior in body fluids and mechanical modulus, can be altered to specific biomedical applications [57]. In a full thickness excisional wound healing mouse model curcumin loaded PLGA was successfully used for wound healing ability [58]. *In vivo* release study in mouse model, using PLGA-Collagen nanofibers with antimicrobial agents exhibit biphasic pattern of drug release [59]. Electrospun copolymer of PCL-PLGA fabricated using different ration proven that biodegradability of electrospun mat increases with increasing PLGA concentration [60].

1.3 The Research Hypothesis

Healing of large and deep cut wound needs tissue adhesives and external sealants to prevent blood loss. Currently various biological glue and synthetic adhesives are clinically used either as an adjunct to conventional hemostasis and wound closure technique or as a replacement to them. The effective control of bleeding reduces the risk of complications associated with severe blood loss. Various synthetic and biological sealants are commercially available for short term need. A biofunctionalized synthetic electrospun matrix with sealant property is proposed for large area rapid sealing. The hybrid material is expected to enable spontaneous sealing and extended duration.

1.4 Research Objectives

1. Electrospinning of biocompatible and biodegradable polymer.
2. Biofunctionalization of electrospun mat with blood coagulating factor, thrombin, and analysis of efficiency.
3. Evaluation of cytotoxicity and cytocompatibility of biofunctionalized electrospun mats.
4. Fabrication of biofunctionalized and Biodegradable electrospun mat for large area tissue defect.
5. Device models of wound dressing with super absorbent property for exuding wounds.

Chapter 2

MATERIALS AND METHODS

2.1 Fabrication of Electrospun PCL Mat

2.1.1 Preparation of PCL Solution

Ten weight percentage solution of PCL (average M_n 80000, Sigma) was prepared by dissolving 1g PCL in 10 ml solvent containing Tetrahydrofuran (THF; Spectrochem) and dimethylsulphoxide (DMSO; Spectrochem) based on previously reported method [61]. The PCL solution was stirred for 2-3 h using a magnetic stirrer before used for electrospinning.

2.1.2 Electrospinning

Non-woven mats of PCL having porous fibers (PPCL) were fabricated by electrospinning using are customized electrospinning unit (Holmarc Opto-Mechatronics Pvt Ltd, India) in a horizontal spinning configuration. The polymer solution was taken in a 10 ml syringe with a blunt end needle and connected to a syringe pump. A potential difference of 12-15 kV was applied using an adjustable high voltage power supply between the needle and a stationary grounded metallic collector. In order to fabricate mats with different mechanical properties, the fibers were captured using a stationary plane and grid collector. Electrospinning was set at a flow rate of 1.5 ml/hr with a tip to collector distance of 12-15 cm. After completing electrospinning the electrospun mats were washed with deionized water (Milli-Q) 2 to 3 times. The cleaned mats were dried in hot air oven set at 37°C overnight.

2.1.3 Sterilization of electrospun mats

Electrospun mat was cut into desired size and continuously washed by using distilled water in a Gel Rocker for 4-5h. After through washing mat was sterilized in 70% ethanol for 20 min and air dried before using

2.2 Physicochemical Characterization electrospun mat

2.2.1 Morphological Characterization

The fibrous mats prepared using plane and grid collectors were observed under Scanning Electron Microscopes (FEI Quanta 200 Environmental Scanning Electron Microscope and FE-SEM, Hitachi 6600). Representative samples were cut from each mat were sputter coated with gold before observing under SEM. The digital images were acquired at various magnifications and data was compared.

2.2.2 Fourier transform infrared spectroscopy (FTIR) Analysis

The surface chemistry was analyzed by determining the functional group of electrospun PPCL mats by FTIR spectroscopy in mid-IR range of 4000-400 cm^{-1} using a Nicolet Inc. (Madison, WI) Spectrometer in the diffused reflectance mode.

2.2.3 Mechanical characterization

Electrospun PPCL mats in plane and grid forms were sized according to ISO 527-2 specimen type-5A for mechanical testing. Samples (n=6) were cut and the sample thickness were measured using thickness gauge. Mechanical analysis was performed in Instron Universal Materials Testing Machine (Instron Corp., India) with 100 N load cells with crosshead speed of 10 mm/min. Tensile strength, stress, strain, Young's modulus, elongation at break was calculated (BLUE HILL 3 computer software).

2.3 Biological Evaluation of PPCL mat

2.3.1 Cytotoxicity analysis

2.3.1.1 *Direct contact*

The cytotoxicity of plane and grid PPCL mat were evaluated using HaCaT (Human Keratinocytes, NCCS Pune, and India). Cells maintained in Minimum Essential Medium (MEM, Sigma) supplemented with 10 % Foetal Bovine Serum (Gibco) and Penicillin-Streptomycin antibiotic (Pen Strep—10,000 U/ml) was enzymatically obtained (0.25 % Trypsin and 0.02% EDTA) in suspension. Approximately 3×10^5 cells were seeded to 24 well plates and allowed to form monolayer at 37 °C in a CO_2 incubator. After removing culture medium, PPCL mats having 4×4 mm size were carefully placed on the cell

monolayer. Poly vinyl chloride and poly ethylene discs were used as positive and negative control. Sufficient culture medium was added and cells were incubated for 24 hrs at 37 °C in a CO_2 incubator. The cytotoxicity was assessed by analyzing the cell morphology, detachment, lysis and vacuolization under an inverted phase contrast microscope (Nikon TS100).

After direct contact test, the viability of cells around test sample was determined by neutral red uptake method. Cells with material was incubated with 0.5 mg/ml neutral red in normal saline for 5 min and observed under microscope in bright field mode.

2.3.1.2 *Test on Extract with MTT Assay*

The potential leachent from the fabricated material that can elicit cytotoxicity was studies by MTT [3-(4,5-Dimethyl thiazol-2-yl)-2-5-diphenyltetrazolium bromide; Sigma] assay. L-929 cells seeded on 96 well plates at density of 1×10^5 cells were allowed to reach subconfluency. Meanwhile the cells attained subconfluency, Test material extract was prepared by incubating 3×1 cm PPCL mats in 1 ml culture medium at 37± 1℃ for 24± 2 h. The extracts were diluted with culture medium to get 50%, 25% and 12.5%. Equal volumes of various dilution of test extract were placed on subconfluent monolayer of L-929 cells. Phenol at a concentration of 13 mg/ml in culture medium was taken as positive cytotoxic control and cell supplied with normal medium was considered as cell control (negative cytotoxic). Cells exposed to various dilutions of extract, positive and negative control were incubated at 37± 1℃ for 24± 2 h. The culture medium was replaced with 50µl MTT solution (1mg/ml) and cells were incubated at 37± 1℃ for 2 h. After discarding the MTT solution 100µl of Isopropanol was added to dissolve the formazan product formed as a result of cell activity. The purple colour developed was quantified by measuring absorbance at 570 nm using a spectrophotometer (Biotek, USA). The optical density obtained for test samples and positive controls were compared with cell control.

2.3.2 Cytocompatibility analysis

2.3.2.1 *Cell adhesion and viability*

The cell adhesion, morphology and viability of cells on PPCL mats were analyzed using HaCaT cells. Approximately, $1x10^5$ cells were seeded on 1x1 cm electrospun PPCL

mat was incubated for 30 min at 37℃ in a CO_2 incubator to ensure cell adhesion. After 30 min, sufficient media was added and incubated for 3 days. The viability of cells adhered on mats were determined by live-dead staining using a combination of fluorescein diacetate (FDA) and propidium iodide (PI)(FDA and PI purchased from Sigma). The cell seeded samples were incubated with 1 ml FDA medium (10 µg/ml FDA in serum free MEM) for 5 min. On a glass slide FDA medium was added and then mixed with 10µl of PI (5µg/ml in PBS) followed by placing the mat with cell side facing down. The adhered cells on the mat were examined under a fluorescence microscope [Leica DMI 6000B equipped with I3 (green) and N21 (red) filter].

The morphology of adhered cells on materials were analyzed by staining actin filaments. Cells adhered on PPCL mats were fixed using 4% paraformaldehyde(Sigma) for 1 h. The cells were rinsed with PBS and permeabilized with 0.1% Triton-X100 for 5min. After removing detergent, cells were rinsed and incubated with phalloidin conjugated with FITC (50 µg/ml, 20 min) purchased from Life Technology. Cell nuclei were counter stained with Hoechst (0.5 µg/ml) for 1min. The morphology of the cells on material was observed under fluorescence microscope.

2.3.2.2 *Cell affinity towards material*

The electrospun PPCL mat in the study is proposed for wound care application. A new test method was adopted to analyze the affinity of cells to adhere on the mats. Keratinocytes were cultured on non-tissue culture treated non-cell adherent plates and the test material was presented on top of settled cells. Being on a non-adherent surface, cell affinity will determine its adhesion towards overlaid material. The HaCaT cells were trypsinized and seeded on tissue culture treated and non-treated culture dishes and allowed to settle for 4 h at 37℃ in a CO2 incubator. PPCL-G, PPCL-P and cover glass having 1×1 cm size were carefully placed on the cells and culture medium was added making sure that underlying cells are in contact with the material placed above and incubated for 72 h. The affinity of cells to adhere on materials was determined by staining the cell nuclei. Cells were fixed using 4% paraformaldehyde and incubated with Hoechst (0.5 µg/ml) for 1 min. The presence of cells on the material was considered to be a positive affinity towards the material.

2.3.3 Hemocompatibility

Blood from human volunteer was collect into the anticoagulant CPD-A. Porous PPCL mat, freshly biofunctionalized PPCL mat, lyophilized biofunctionalized PPCL mats in triplicate were placed in polystyrene culture plates. One and added 1 ml PBS and were immersed in PBS and agitated for 5 min. PBS was aspirated out and 1.5 ml of blood was added. Immediately, 0.5 ml blood was taken for initial analysis and the sample was incubated with remaining 1 ml blood for 30 min under agitation at 70 ± 5 rpm using an Environ shaker thermo stated at 35 ±2 °C. Polystyrene surface exposed to blood was considered as control.

Quantification of platelet adhesion and percentage hemolysis were calculated for each sample. Quantification of platelet adhesion was analyzed in initial and 30 min blood samples using Haematology Analyzer (Sysmex-K). Percentage change was calculated from the initial and final readings. The hemolysis was measured by analyzing the total hemoglobin in the whole blood samples were measured using automatic hematology analyzer (Sysmex-K 4500). The percentage hemolysis was calculated by the difference between the measurements of the whole blood and the blood exposed to samples using Diode array Spectrophotometer. The percentage hemolysis was calculated using the formula (Free Hb/Total Hb/1000) x 100.

2.4 Modifications of the Electrospun PPCL mat with thrombin

Biofunctionalization of PPCL mats for rapid blood clotting was achieved by coating it with Bovine thrombin (Sigma). The PPCL mats biofunctionalized with thrombin was coded as TBfPPCL. The biofunctionalization was optimized by applying fixed and variable thrombin concentration to a constant surface area. Thrombin was prepared by dissolving thrombin in PBS to a final concentration of 1 IU/µl.

Constant thrombin concentration: 5 µl of thrombin (5 IU) was evenly coated on PPCL mat having size of 1×1 cm using a micropipette.

Variable thrombin concentration: Different concentration of thrombin solution was prepared by diluting with PBS. Equal volume (5 µl) of variable thrombin concentrations such as 5 IU, 2.5 IU and 1.25 IU was evenly coated on PPCL mat having size of 1×1 cm using a micropipette.

2.4.1 Efficacy of Biofunctionalization

The efficiency of biofunctionalization of PPCL was determined by evaluating the clot formation in the presence of plasma. The experiment was conducted using TBfPPCL-P in two sets. In the first set, mats biofunctionalized with fixed concentration of thrombin (5 IU) were exposed to different volume of plasma (25 µl, 50 µl and 100 µl) at room temperature for 20 min. The second set contained mats biofunctionalized with different concentration (5 IU, 2.5 IU and 1.25 IU) of thrombin exposed to fixed volume of plasma (100µl) at room temperature for 20 min. The samples were rinsed with PBS to remove free plasma proteins from the clot formed. The fibrin clot was precipitated in 1 ml of 10% TCA (Trichloroacetic acid, Sigma) solution and centrifuged at 10000 rpm for 10 min at 4℃. Supernatant was discarded and the centrifugation step was repeated with fresh TCA. The pellet was dissolved in 1 ml ice cold acetone and kept at -20℃ for 1 h with intermittent vortexing for 30 sec in every 15 min. The precipitate was centrifuged at 10000 rpm for 10 min at 4℃ and the pellet was air dried.

2.4.1.1 *Protein estimation*

The clot formation was analyzed by estimating the protein using Bio-Rad *DC* protein assay kit (Lowry's method) according to manufacturer's instructions. The sample was prepared by dissolving protein pellet from fibrin clot in 1N NaOH. Bovine serum albumin (BSA, Sigma) was used as standard (2.5 mg/ml, 2 mg/ml, 1.5 mg/ml, 1 mg/ml, 0.5 mg/ml) to plot the standard curve. To 5 µl test and standard samples taken in a 96 well plate, 25µl reagent A and 200µl reagent B was added. The color developed was quantified by analyzing the absorbance at 750 nm at room temperature.

2.4.1.2 *Protein characterization*

The clot formed during exposure of TBfPPCL was analyzed by reducing sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE). Protein pellet was dissolved in 1X SDS gel loading dye and placed in water bath at 75°C for 10 min. The proteins were electrophoresed using 10% resolving and 5% stacking gel. 5µl Marker, 10 µl of samples was loaded into wells. A voltage of 100V for stacking gel and 120 V for resolving gel was applied till until the dye front reached the bottom of the gel. The resolved proteins were stained with Coomassie Brilliant Blue solution for staining 20 min.

After rinsing the gel with Milli-Q water, excess stain was removed by place in destaining solution to obtain clear protein bands.

2.4.2 Standardization of Biofunctionalization

The biofunctionalization of electrospun mats were standardized by measuring the total protein of the clot formed in the presence of plasma at different time points. TBfPPCL-P mats were incubated with 100 µl plasma at room temperature for different time period such as 5 min, 10 min, 15 min, 20 min, 40 min and 60 min. Clot formed at each time points were extracted in TCA protein precipitation and done protein estimation by Lowry's method using Bio-Rad *DC* protein assay kit.

2.4.3 Effect of storage conditions on Biofunctionalized PPCL mat

The shelf life of biofunctionalized PPCL mat was analyzed at different time and storage conditions.

The TBfPPCL-P (1×1 cm) functionalized with 1.25 IU thrombin was exposed to plasma and efficiency to form clot were analyzed within 5 min (freshly prepared) and after 24 h storage at room temperature (27 °C) and freezer (0 °C). To improve the storage conditions, the functionalized samples of PPCL-P and PPCL-G were also tested 10 days after lyophilization (LTBfPPCL-P and LTBfPPCL-G). The effect of storage was assessed by estimating the clot formation upon exposure to plasma was estimated. The clot was rinsed with PBS followed by precipitation of protein with TCA and estimated the concentration using Bio-Rad *DC* protein assay kit.

2.4.4 Biological evaluation of biofunctionalized PPCL mats

The cytotoxicity of plane and grid PPCL mat were evaluated using L929 (mouse subcutaneous fibroblasts, ATCC USA) and HaCaT (Human Keratinocytes, NCCS Pune, India). Cells maintained in Minimum Essential Medium (MEM) supplemented with 10 % Foetal Bovine Serum and Penicillin-Streptomycin antibiotic was enzymatically obtained (0.25 % Trypsin and 0.02% EDTA) in suspension. Approximately 3×10^5 cells were seeded to 24 well plates and allowed to form monolayer at 37 °C in a CO_2 incubator.

Cytotoxicity of TBfPPCL-P, TBfPPCL-G, LTBfPPCL-P and LTBfPPCL-G were evaluated by direct contact using L929 (mouse subcutaneous fibroblasts, ATCC USA) and HaCaT (Human Keratinocytes, NCCS Pune, India). The test on extract with MTT assay

was also conducted using L-929 cells to study the potential leachant from the samples. Cytocompatibility was determined using HaCaT by analyzing cell adhesion and viability and cell affinity towards materials. Hemocompatibility was also assessed by exposing materials to blood as described before.

2.4.5 *Ex vivo* wound model

An ex vivo bleeding skin model was designed to evaluate the efficiency of biofunctionalized PPCL mats. Rabbit skin was collected from animals sacrificed for other experiments (SCT/IAEC-060/AUGUST/2013/81). The skin was cleared from hair and spread on a non-wetting surface with the epidermal side facing top. A large area partial thickness wound was simulated by removing a flap of skin from the top. A syringe was connected to the skin such that the blood or plasma taken within it flows into the wound site as the piston moves forward. Equally large TBfPPCL-P mats were placed over the bleeding *ex vivo* wound and the clotting time was monitored. The clot formed by perfusion of plasma and blood was analyzed from the wound surface and the TBfPPCL-P was estimated to see the clot formation. The clot formation was confirmed by analyzing the clot by reducing SDS-PAGE.

2.5 Biofunctionalized biodegradable electrospun mat as large area tissue sealant

After confirming the efficiency of PPCL as potential sealant by biofunctionalization with thrombin, a biodegradable biocompatible electrospun mat was fabricated by blending PCL with PLA/PGA RESOMER® [Poly (D, L-lactide-co-glycolide); PLGA 50:50; RESOMER®, Sigma]. The PLGA polymer has in vivo degradation time of 14 days and hence is expected to degrade within the wound site. A blend of PCL and PLGA polymer was fabricated on a PCL mat. A thin layer of PCL mat was fabricated on a stationary collector followed by electrospinning PCL/PLGA blend solution (0.2 g PCL and 1g PLGA in 2 ml THF: DMF solvent).

2.5.1 Biofunctionalization of PCL/PLGA mat and Determination of its efficiency.

Biofunctionalization of PLGA-PCL mats for rapid blood clotting were achieved by coating it with Bovine thrombin (Sigma). The PCL/PLGA mats biofunctionalized with thrombin was coded as TBfPCL/PLGA. 5 µl of thrombin (1.255 IU) was evenly coated

on mat having size of 1×1 cm using a micropipette. The efficiency of biofunctionalization was determined by evaluating the clot formation in the presence of 100 µl of plasma. After 20min of clotting time the samples were rinsed with PBS to remove free plasma proteins from the clot formed. The fibrin clot was precipitated in 1 ml TCA (10%) solution and centrifuged at 10000 rpm for 10 min at 4℃. Supernatant was discarded and the centrifugation step was repeated with fresh TCA. The pellet was dissolved in 1 ml ice cold acetone and kept at -20℃ for 1 h with intermittent vortexing for 30 sec in every 15 min. The precipitate was centrifuged at 10000 rpm for 10 min at 4℃ and the pellet was air dried. The clot formation was analyzed by estimating the protein using Bio-Rad *DC* protein assay kit (Lowry's method) according to manufacturer's instructions.

2.5.2 In vitro degradation of PCL/PLGA mat

In vitro degradation of PCL/PLGA electrospun mat in Simulated Wound Fluid (142 mM NaCl, 2.53 mM $CaCl_2$, 5% BSA) was analyzed. Samples having 1×1 cm size was incubated with 10 ml SWF at 37℃ with continuous agitation. Weight loss was analyzed on 7^{th} and 14^{th} day. The percentage weight loss was calculated from the equation (Final Weight/Initial Weight) ×100.

2.6 Device models using PPCL electrospun mat

2.6.1 Modification of Electrospun mat for fluid absorption

A wound dressing device which can absorb fluid exudates was developed from PPCL-P mats. The well known superabsorbent polymer, Sodium polyacrylate was used to impart fluid absorption property to the proposed wound dressing. Absorbent gauze containing super absorbent polymer (SAP) was sandwiched between two PPCL-P mats and the sides were heat sealed. The super absorbent dressing thus formed was considered as super absorbent bandage (SA-Band).

2.6.1.1 *Fluid absorption of SAP*

10 mg sodium polyacrylate was weighed in to two separate dishes. 2 ml water was added to one plate and 2ml anti-coagulated blood to another plate. After 5 minutes of incubation at room temperature excess blood and water was removed. Final weight was taken to analyzed to determine the fluid absorption efficiency of sodium polyacrylate.

2.6.1.2 *Fluid absorption by SA-Band*

Cotton gauze of 2×2 cm size containing 100 mg SAP was sandwiched between two PPCL-P mats. Fluid absorption ability was studied using anti coagulated blood and water. The SA-Band was placed over 1.5 ml fluid and observed for absorption and duration.

Chapter 3

RESULTS AND DISCUSSION

3.1 Fabrication of Electrospun PPCL Mat

Poly(ε-caprolactone) (PCL) is an FDA-approved biodegradable polymer which has attracted increasing interest in biomedical application due to its excellent biocompatibility and flexibility [62]. PCL is a bioresorbable polyester and the degradation products eliminated naturally by the metabolic pathways avoiding the possibility of foreign body reaction [63]. PCL has been extensively used in many biomedical applications including skin [64]. One of the most intensively studied for developing biomaterials medical application is nanofibers fabricated by electrospinning technology [45]. Electrospinning is a process that produces polymer solution spun under high potential electric field to yield non woven fibers with diameters ranging from nano or micro size. A charged jet is accelerated towards a grounded collector. The charged polymeric molecules repel each other within the jet together and the solvent evaporation causes the formation of fibers. The property of large surface area to volume ratio makes the electrospun matrix as an excellent candidate for wide range of application including biomedical application [65]. Polycaprolactone fibers was fabricated by electrospinning 10% PCL solution and collected on stationary grounded plane and Grid collector. It has been reported that the electrospun fibers become porous when a binary solvent system is used due to the non-solvent phase separation mechanism [66]. In this study tetrahydrofuran and dimethyl sulfoxide was taken as a binary solvent system for fabricating PCL mats with porous fibers – PPCL (Figure 1). The mat formed on plane collector (PPCL-P) was random nonwoven type with tightly packed fibers. However the fibers collected on metallic grid (PPCL-G) resulted in a mesh like pattern. The PPCL-G was easy to handle because of its reinforced structure compared to PPCL-P.

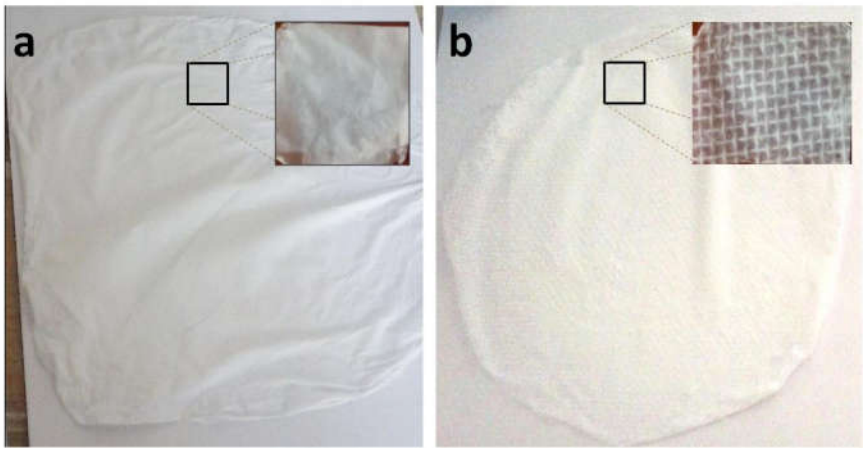

Figure 1. PPCL Electrospun mats collected on a) plane collector and b) Grid collector.

3.2 Physicochemical Characterization electrospun mat

3.2.1 Morphological characterization

Nanofibers remain to be a new class of material in spite of the rapid development in recent years. The enormous amount of literature shows that it is essential to consider the detailed characterization in addition to the preparation methods. Among the various features of nanofibers such as chemical composition, mechanical properties, thermal behavior or hydrophilicity, the parameter which has a key role is the fiber morphology, especially in biomedical field [67, [68]. The architecture of nanofibrous materials has been reported to be a good candidate as supporting materials in cell therapy including chronic wounds [69]. In this study laboratory optimized in-house developed procedure was used to fabricate electrospun mat having porous fibers. The fiber morphology was analyzed under high magnification using SEM. The PPCL-P and PPCL-G mats showed bead free fibers with uniform fiber thickness (Figure 2).

The electrospinning process gives randomly aligned fiber mesh as end product. The stretching exerted by the electric field creates the random deposition. However the fibers can be aligned to a particular pattern by using collectors with definite dimension and designs [70]. The patterns can provide fiber meshes with specific architecture suitable for biomedical applications.

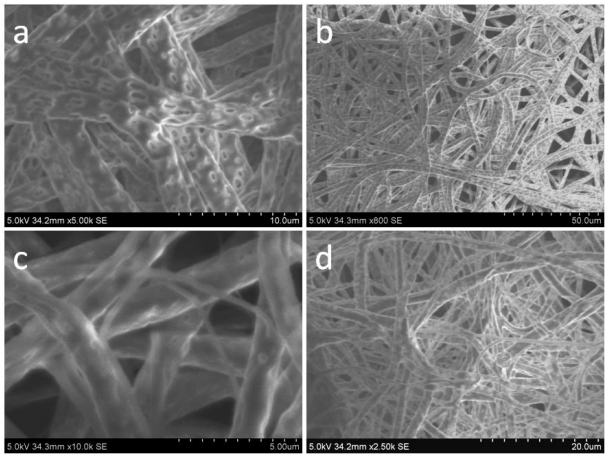

Figure 2. SEM images showing PPCL-P and PPCL-G mat. (a&c) The fibers expressed pores throughout the length with uniform distribution. (b&d) The mat expressed closely packed random fibers.

To improve the mechanical property of the electrospun mat, patterned electrospun fiber layer was prepared by using grid as collector. The PPCL-P fibers were random and tightly packed whereas the PPCL-G on grid expressed mesh pattern. The fibers on the wires of grid were aligned where as the space between the wires were filled with random (Figure 3). The results confirmed that the procedure for fabricating PPCL mats was optimum to yield similar characteristics in more than 12 batches. The findings are also similar to those previous report [66]

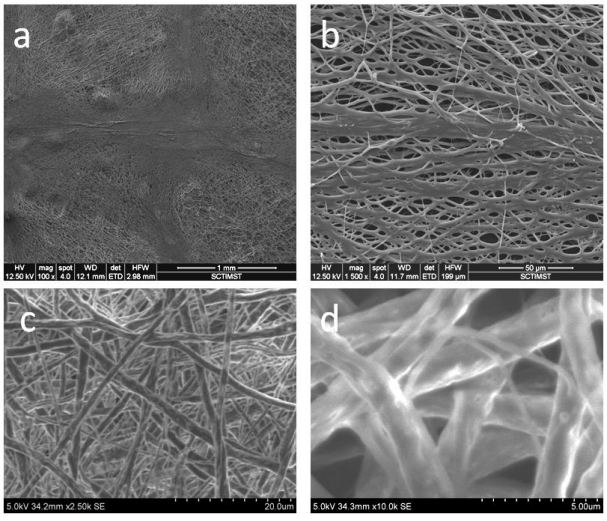

Figure 3. PPCL-G mat observed under SEM. (a) Grid pattern of mat, (b) The aligned fibers on grid wires, (c) random fibers between gird and (d) pores on the fiber under higher magnification

3.2.2 IR Analysis

FTIR spectroscopy is a technique used to collect data in the form of infrared spectrum in order to analyze and characterize the sample. This spectrum provides information about the functional groups that forms the materials. Absorbance of electromagnetic radiation in the infrared range is based on the vibrational and rotational energy levels in bond present in the functional groups. FTIR also used to study the polymerization reaction [71, [72]. According to Figure-2 typical band for PCL are asymmetric CH2 stretching at 2942cm^{-1} , Symmetric CH2 stretching at 2864 cm^{-1} , Carbonyl stretching at 1722 cm^{-1} , C-C and C-O at crystalline phase at 1293 cm^{-1} and asymmetric C-O-C stretching at 1240 were observed. This results are shown to be same as that of previous report [73].

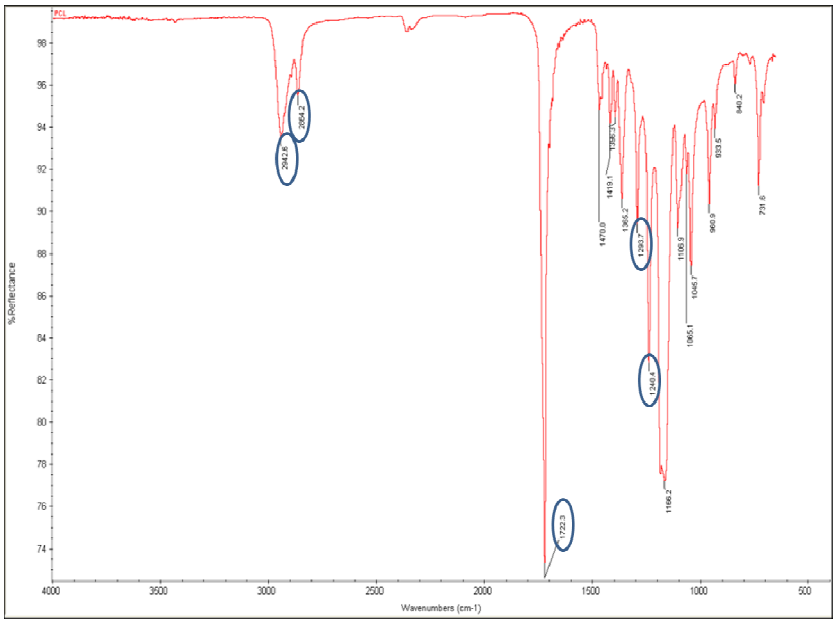

Figure 4. FTIR Spectra of PPCL

3.2.3 Tensile strength

PPCL-P and PPCL-G mats were analyzed for mechanical properties in an Instron tensile tester. Tensile strength of PPCL mat fabricated by using plane and grid collector was measured. Tensile strength expresses the maximum stress developed in dressing material during tensile testing [74]. For an ideal wound dressing substrate, the materials must have good tensile strength to avoid damage due to handling. The Young's modulus and strain at break were calculated from the stress–strain curves. The data obtained are presented in

Table 1. The PPCL-P showed Young's Modulus of 7.2 MPa and average value of elongation at break of 104.9 %. On the other hand the PPCL-G mats expressed Young's Modulus of 7.5 MPa and elongation at break at 302.5 %. Croisier et al reported that normal PCL electrospun mat shows Tensile strength around 3 MPa [75]. The modulus of plane PPCL (PPCL-P) is only one third of this reported value. This could be due to the pores present on the fibers. However the grid pattern of PPCL-G mats showed improved tensile property of 2 MPa could be due to the reinforcement effect of the design and the porous and non-porous fibers. The PPCL-G showed typical PCL stress-strain curve as previously reported.

Table-1

Materials	Thickness (mm)	Tensile strength (Mpa)	Elongation at break (%)	Young's Modulus (Mpa)
PPCL-G	0.256	2.094	302.536	7.460
PPCL-P	0.048	1.630	104.885	7.252

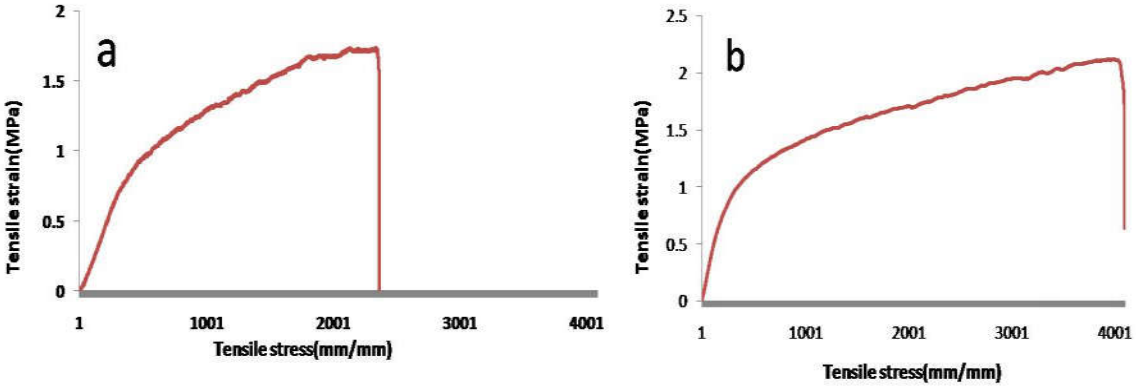

Figure 5. (a) Stress-Strain curve of PPCL-G (b) Stress-Strain curve of PPCL-P.

3.3 Biological Evaluation of PPCL mat

3.3.1 Cytotoxicity analysis

3.3.1.1 *Direct contact*

Cytotoxicity is a preliminary test required to verify the potential toxicity response to biological system. Cytotoxicity by direct contact method yields direct contact of the solid medical devices with cultured mammalian cells and the response of cells towards device materials ([76]). *In vitro* Cytotoxicity test by direct contact method was performed

using HaCaT cells. The cells around PPCL-P and PPCL-G after 24 h contact revealed non-cytotoxic nature. The toxic responses like cell detachment, lysis and extensive vacuolization were absent similar to negative control (Figure 6 a, b and c). The viability of the cells after direct contact test was further confirmed by analyzing the viability by neutral red staining (Figure 6 e, f and g). The positive control showed severe cytotoxic response (Figure 6 d and h). The results confirmed that the PPCL mats fabricated using plane and grid collectors were none cytotoxic to keratinocytes. This confirms the initial safety screening of the materials for wound dressing application and the data obtained from direct contact of cells with materials are comparable with previously reported data [77].

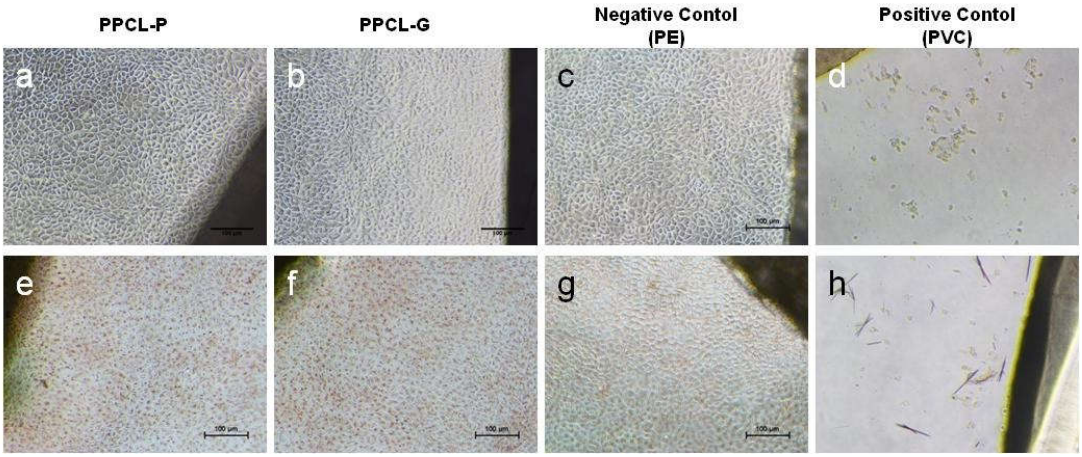

Figure 6. Direct contact of HaCaT cell with PPCL-P and PPCL-G.

3.3.1.2 *Test on Extract with MTT Assay*

(3-(4,5-dimethyl-2-thiazolyl)- 2,5-diphenyl-2H-tetrazolium bromide) assay is also known as mitochondrial dehydrogenase performance measurement because this enzymes present in living cells cleave the tetrazolium ring present in MTT reagent as a result yellow water soluble MTT reagent is reduced to form a purple crystalline formazan. Amount of formazan crystals formed is the reflection of number of living cells. This test is used to assess cell proliferation and cytotoxicity through colorimetry to measure cell metabolism [78]. Cytotoxicity analysis by test on extract according to ISO 10993-5 and ISO 10993-12 is widely used for *in vitro* biocompatibility study [79, [80, [81]. Test on extract assay provides potential toxicity due to soluble leachant from biomaterials including wound dressing bandages [82]. The extract of PPCL-P and PPCL-G was

obtained in culture medium with serum at a ratio of 6 cm^2/ml. L-929 cells exposed to various dilutions of extract for 24 h showed normal cell growth and morphology. The metabolic activity of the cells exposed to extracts of materials was assessed by MTT assay. The 100 % extract of PPCL-P and PPCL-G showed 75 ± 3 % and 78 ± 7 % cell activity respectively compared to cells cultured in normal medium (Figure 7). However as the dilution increased the cell activity was also increased indicating possible leachant or other components from the mat. The positive control used was phenol with confirmed cytotoxicity.

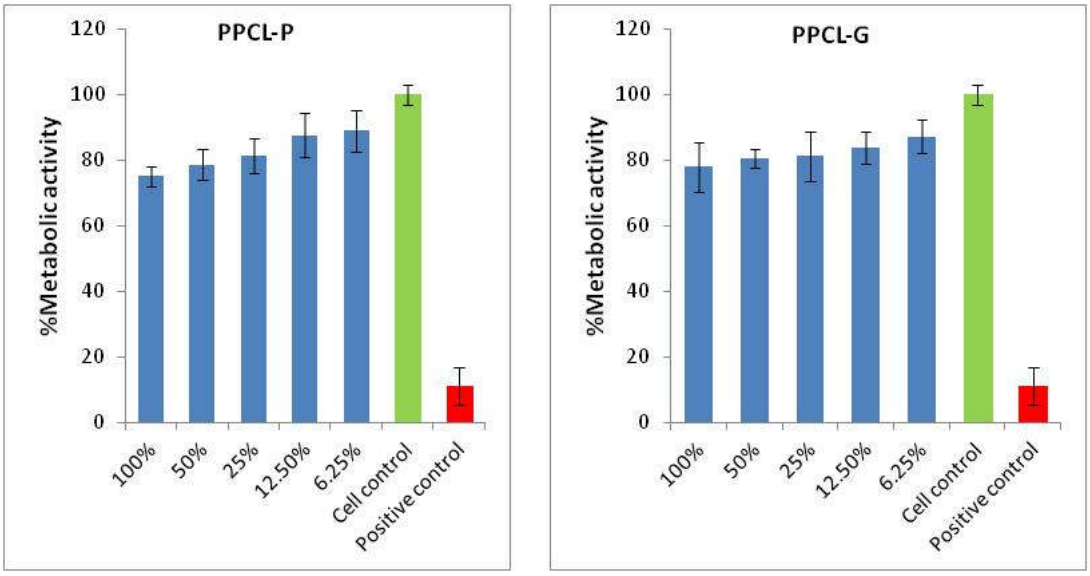

Figure 7. The MTT assay of PPCL-P and PPCL-G mats .

Hence the method of fabrication to sterilization was rechecked and additional washing steps were included. The test was repeated with electrospun mats and the 100% extract showed cell activity similar to the cell control (Figure 8) indicating the non cytotoxic nature. This confirmed the fabrication steps followed to be complete to give non cytotoxic electrospun mats for wound dressing application.

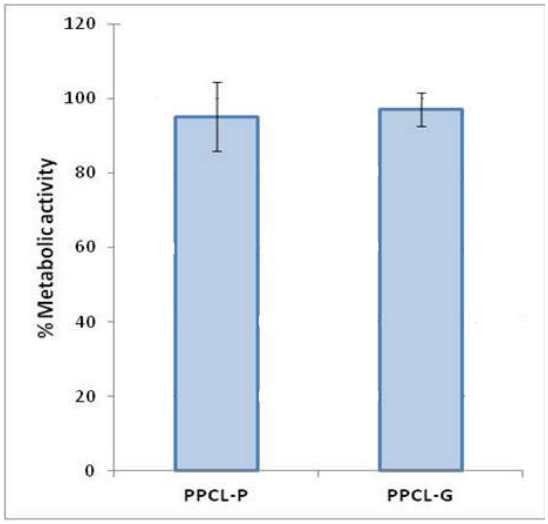

Figure 8. MTT assay of PPCL-P and PPCL-G after additional washing step.

3.3.2 Cytocompatibility analysis

3.3.2.1 *Cell adhesion and viability*

The interaction of cells to biomaterial is possible when the cell surface receptors interact with the cell binding domain of extra cellular matrix proteins [83]. The surface topography, wettability, protein adhesion, surface charge and surface roughness significantly influences cell behaviors such as adhesion, spreading and proliferation [84]. In normal Tissue culture plates cell adhesion is provided by growth factors present in the serum. HaCaT cells cultured on PPCL-P and PPCL-G confirmed that the cell adhesion is normal on the material. The cells expressed characteristic polygonal morphology when stained for cytoskeletal actin filaments. The cells also exhibited the patched growth pattern characteristic to HaCaT cells [77, [85].

The viability of adhered keratinocytes on PPCL mats were visualized by FDA-PI staining. Viable cells showed green color where as nucleus of dead cells appeared red in color. This results compiled with previous report [86] and most of the HaCaT cells on PPCL-P and PPCL-G were viable (Figure 10).

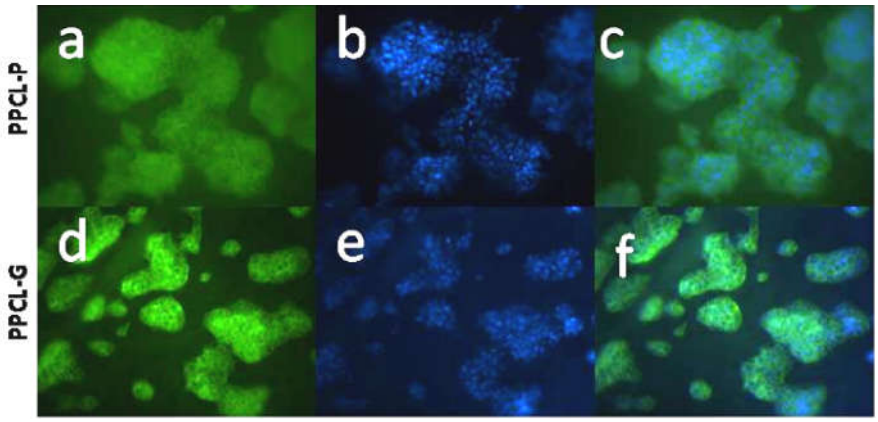

Figure 9. Cell adhesion and morphology of HaCaT on PPCL mats.

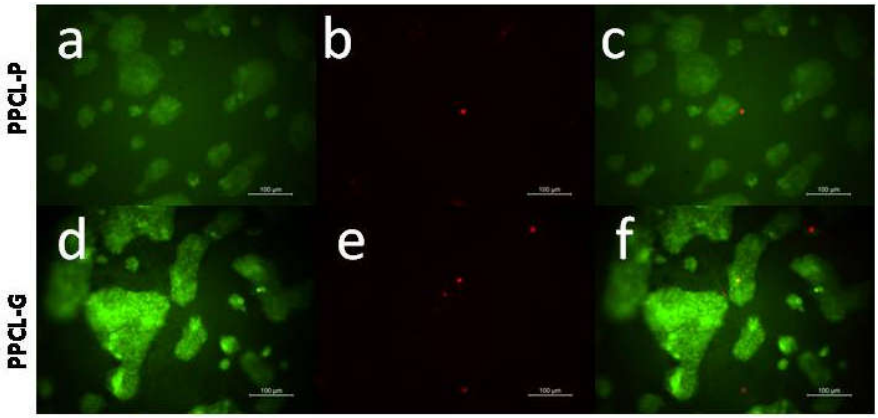

Figure 10. Viability of HaCaT cells on PPCL mat.

3.3.2.2 *Cell affinity towards PPCL Electrospun mat*

The initial adhesive interaction between the cells and the substrate is followed by receptor-ligand binding to enhance the adhesion strength. The cell adhesion process can be classified into three phases like initial attachment, spreading and reorganization of actin skeleton [87]. The electrospun mats in the study is proposed for wound dressing application. If the cells adhered firmly to the dressing materials it would be undesirable in the final application. A new test method was adopted to prove the cell "affinity", the selectivity of cells, towards PPCL-P and PPCL-G. To ensure that the electrospun mats promoting cell adhesion have a lower affinity to keratinocytes, and experimental model was designed as illustrated in Figure 11.

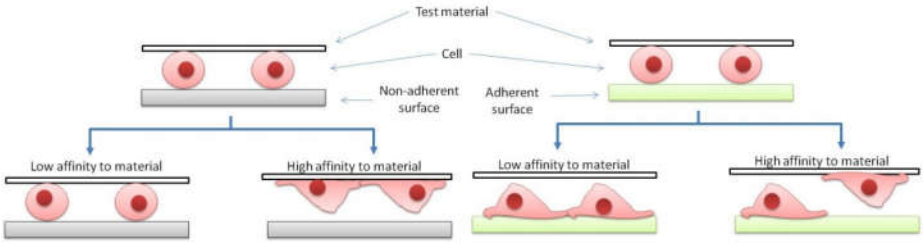

Figure 11. Illustration of the proposed experimental design to determine the affinity of cells towards a material.

In this study cover glass was used as a control for cell affinity for adhesion. HaCaT cultured on non-treated tissue culture dishes remained non adherent. Keratinocytes seeded on normal tissue culture treated adherent surface and placed PPCL-P and PPCL-G mats were placed above, the cells could not attach to the mat. The cells were also not able to adhere on the cover glass placed above indicating the affinity of cells towards glass is lesser than that of tissue culture treated plate. When cells cultured on non-adherent dishes and presented with electrospun mats above, the cells did not adhered on the mats. However the cells adhered on the cover glass as it is a good substrate for cell adhesion compared to non-adherent surface. This clearly indicates the low cell affinity for cell adhesion to PPCL-P and PPCL-G. Since the proposed matrix is biofunctionalized for clotting, the fibroblast in the wound bed will be separated by the clot formed. The cells that directly come in contact with mat will be surrounding keratinocytes and hence keratinocytes were selected for the cell affinity analysis instead of fibroblast.

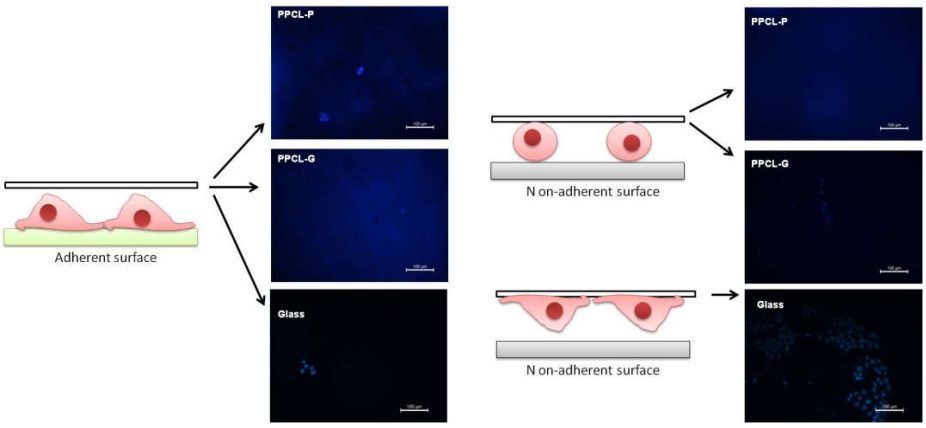

Figure 12. The material overlay method for cell affinity testing.

3.3.3 Hemocompatibility of PPCL mats

When a biomaterial comes in contact with blood, proteins gets adsorbed on the material surface followed by adhesion of platelets and its activation. This eventually leads to thrombus formation [88]. To avoid thrombus formation and improve the blood compatibility surface modifications strategies have to be adopted to alter the hemocompatibility of biomaterial surfaces [89].

Hemolysis represents the free hemoglobin released as a result of breakage of red blood cells when a material comes in contact with blood. When a polymeric devices contact with the blood from a living body number reactions take place so *in vitro* blood compatibility testing of material is an important property to avoid most of the adverse reactions. Hemocompatibility of the PPCL-P and PPCL-G were evaluated by percentage of hemolysis and percentage change of platelet count. The hemolysis percentage (HP) represents the extent of red blood cells broken by the sample in contact with blood. This experiment shown that hemolysis percentage PPCL-P, PPCL-G, were around 0.1% (Figure 13a). According to ISO 10993 part 4 hemolysis should be less than 0.1% but according to ASTM F756-00(2000) standard the percentage hemolysis less than 2% this indication of material with non-hemolysis and good hemocompatibility [90]. Percentage changes of platelet count of PPCL-P &PPCL-G were 25% and 13% (Figure 13b) respectively. According to ISO standard normal platelet adhesion for biomaterial is 10% but here PPCL mats are proposed for wound healing, high Platelet adhesion and aggression are the important properties which enhances blood coagulation [91].

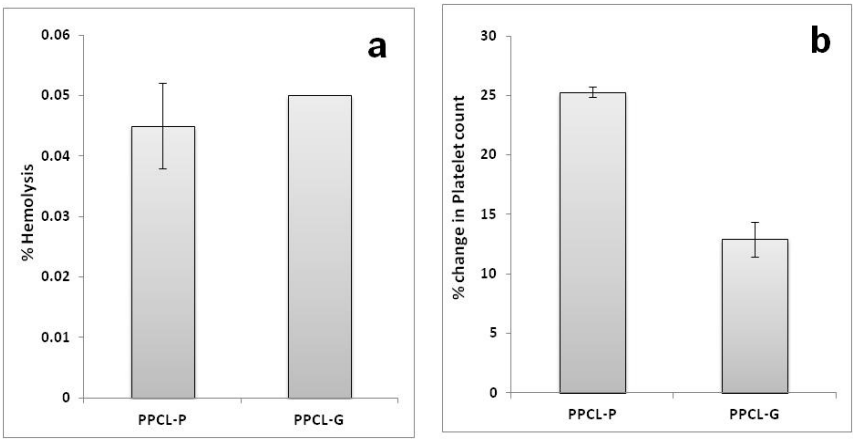

Figure 13. Hemocompatibility evaluation of PPCL mats

3.4 Modifications of the Electrospun PPCL mat with thrombin

3.4.1 Efficacy of biofunctionalization by protein estimation

Biofunctionalization is the modifications of materials to have a biological function. Because of slow biodegradation and hydrophobicity bio-functionalization of PCL matrix create an ideal microenvironment for tissue growth and regeneration. Biofunctionalization can be achieved either through mixing of bioactive agents in polymer solution before electrospinning or surface modification by applying bioactive agents on the surface of mat after electrospinning [92].

To overcome the limitations of wound dressings applied on bleeding wound the PCL electrospun mat was biofunctionalized with thrombin. Thrombin is a key enzyme in coagulation pathway that it converts fibrinogen to fibrin [93]. The efficiency of biofunctionalization of PPCL-P was assessed by analyzing the formation of fibrin clot when the samples were exposed to human plasma.

The analysis was done in two sets – 1) fixed concentration of thrombin vs. variable volume of plasma and 2) Fixed volume of plasma with variable concentration of thrombin In the first set when different volume of plasma was applied on TBfPPCL functionalized with 5 IU thrombin, the clot formation was variable. The amount of clot formed increased with increased in volume of plasma (Figure14b). When the mats functionalized with variable volume of thrombin and exposed to equal volume of plasma, it was observed that a minimum of 1.25 IU is sufficient to form plasma clot (Figure 14a). Moreover the clot formation in the presence of varying thrombin concentration such as 5 IU, 2.5 IU, 1.25 IU and 100 µl plasma was similar so 1.25IU was used in remaining experiments.

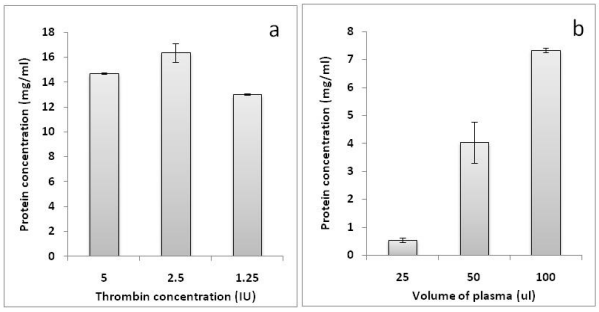

Figure 14. Efficiency of Biofunctionalization by protein estimation.

3.4.1.1 *Protein characterization by SDS-PAGE*

Electrophoresis separation of the protein is accomplished using polyacrylamide gel electrophoresis in which proteins are driven by applied current through a gelated matrix. In SDS-PAGE Polyacrylamide gel electrophoresis carried out in the presence of negatively charged detergent Sodium dodecyl sulfate this allow separation of protein on the bases of single property-their molecular mass [94]. The material prepared from PCL by electrospinning is proposed as a hemostatic agent. Hence the PPCL-P and PPCL-G mats were biofunctionalized with thrombin. The effect of biofunctionalization on blood clotting was assessed by exposing the TBfPPCL-P and TBfPPCL-G to plasma and measuring the clot formed. It was observed the 1.25 IU thrombin to 1×1 cm mats was sufficient enough for clot formation. The clot analyzed by SDS PAGE confirmed that the fibrin clot was formed when the biofunctionalized mats were exposed to plasma. This was evidenced by the presence of band at 50 – 75 KDa [95].

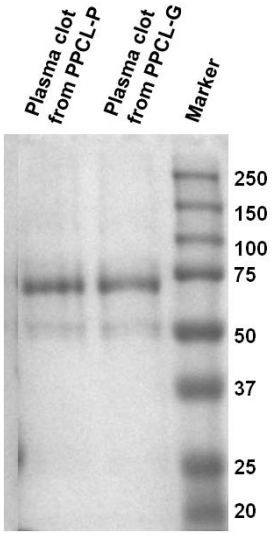

Figure 15. SDS-PAGE analysis of Fibrin clot from PPCL mats

3.4.2 Standardization of Biofunctionalization

Biofunctionalization efficiency of mat was standardized by estimating the concentration of total protein of the clot. The fibrin protein formed on TBfPPCL-P mats at different time points were analyzed. It was observed the clot formation was initiated within 5 min and the maximum concentration of clot proteins were observed at 20 min and 60 min after exposure to plasma (Figure 16).

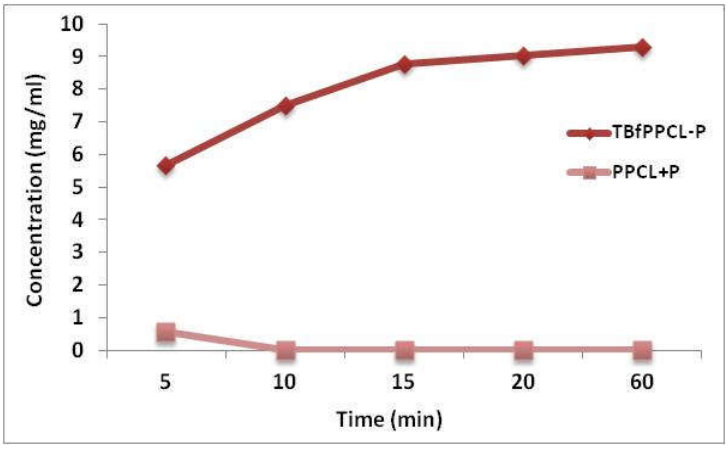

Figure 16. Standardization of Biofunctionalization over time.

3.4.3 Effect of storage conditions on Biofunctionalized PPCL mat

The stability of biofunctionalized PPCL mats was analyzed by estimating the clot proteins within 5 min (27C 5m) and after 24 h (27C 24h) storage at room temperature. The stability was also analyzed after storing TBfPPCL-P mats at 0°C for 24 h (0C 24h) (Figure 17a). The storage duration of the TBfPPCL-P and TBfPPCL-G was lyophilized and the clot formation was analyzed after 10 days. There were no difference in clotting efficiency of Lyophilized and stored sample to freshly prepared mats (Figure 17b).

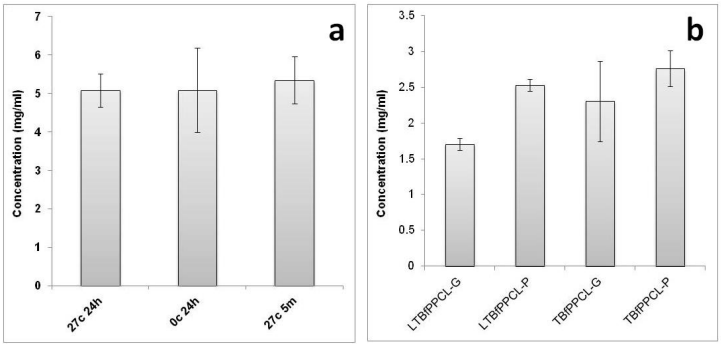

Figure 17. Storage conditions of biofunctionalized PPCL mats.

3.4.4 Biological evaluation

Cytotoxicity of TBfPPCL-P, TBfPPCL-G, LTBfPPCL-P and LTBfPPCL-G were evaluated by direct contact method using L929 and HaCaT cells. The potential leachants was tested by elution method followed by MTT assay. Cytocompatibility tests included the adhesion, viability and the cell affinity of keratinocytes. Hemocompatibility was

analyzed by checking the hemolysis and platelet adhesion after exposing materials to blood.

3.4.4.1 *Cytotoxicity analysis by direct contact test method*

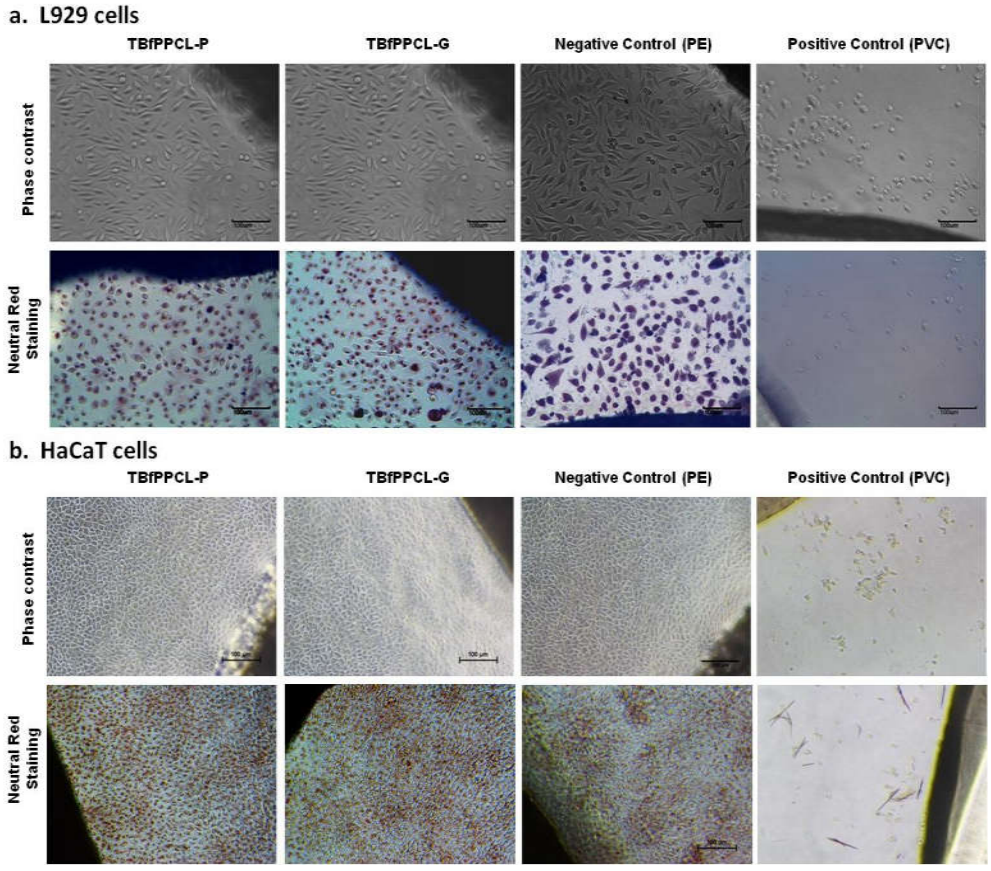

Figure 18. Direct contact cytotoxicity analysis of TBfPPCL-P and TBfPPCL-G with L-929 and HaCaT cells.

The cytotoxicity analysis by direct contact method showed that TBfPPCL-P and TBfPPCL-G are non-cytotoxic to L-929 and HaCaT cells (Figure 18). The morphology of cells around materials after 24 h direct contact was normal and similar to Polyethylene (PE) negative control. Cell detachment, lysis and extensive vacuolization were absent in both TBfPPCL-P and TBfPPCL-G tests.

The viability of the cells confirmed after direct contact test by neutral red staining also revealed that the cells around material was viable. The positive control showed severe cytotoxic response. The results confirmed that biofunctionalization using thrombin on the mats fabricated using plane and grid collectors were none cytotoxic to

general cells (fibroblasts) and specific cell type (keratinocytes). This confirmed the initial safety screening of TBfPPCL-P and TBfPPCL-G as hemostatic material.

To improve the shelf storage, TBfPPCL-P and TBfPPCL-G were lyophilized. The samples stored for more than 30 days were analyzed for cytotoxicity by direct contact method. The results confirmed that the lyophilized (LTBfPPCL-P and LTBfPPCL-G) samples were non cytotoxic to fibroblasts and keratinocytes.

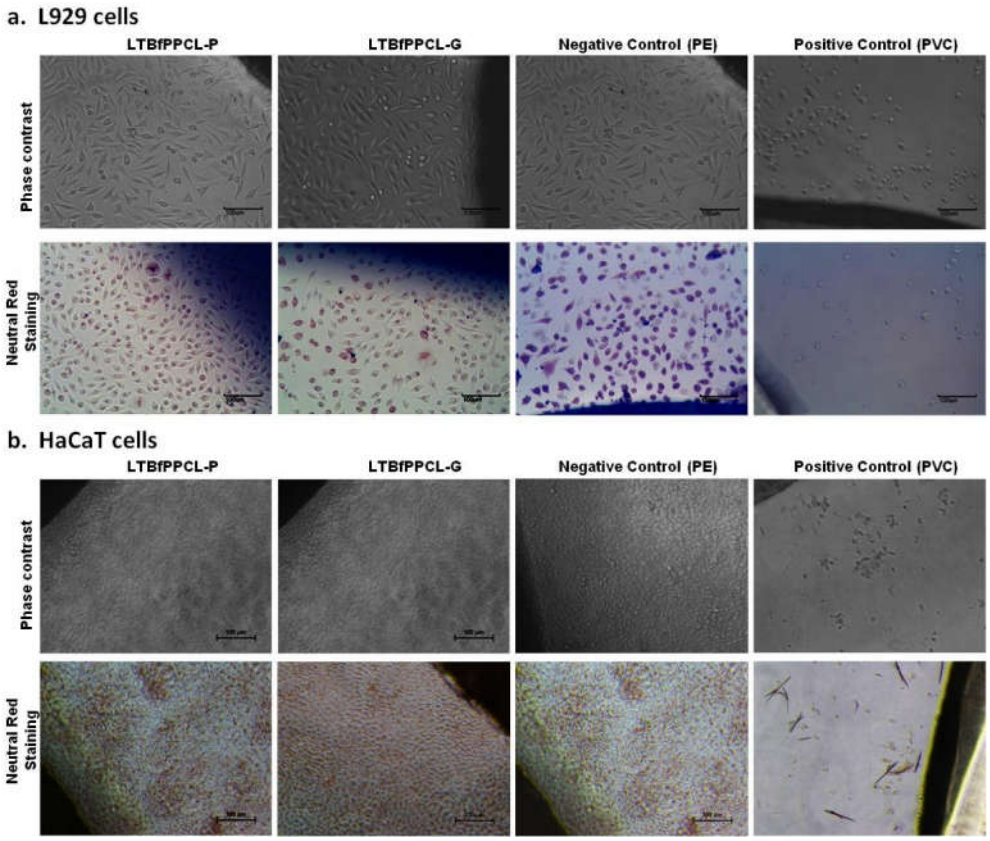

Figure 19. Direct contact cytotoxicity analysis of LTBfPPCL-P and LTBfPPCL-G with L-929 and HaCaT cells.

3.4.4.2 *Cell adhesion and viability on biofunctionalized mats*

HaCaT cells cultured on PPCL-P and PPCL-G confirmed that the cell adhesion is normal on the material. The cells expressed characteristic polygonal morphology when stained for cytoskeletal actin filaments. The cells also exhibited the characteristic polygonal morphology with patched growth pattern on TBfPPCL and LTBfPPCL mat from grid and plane collector (Figure 20).

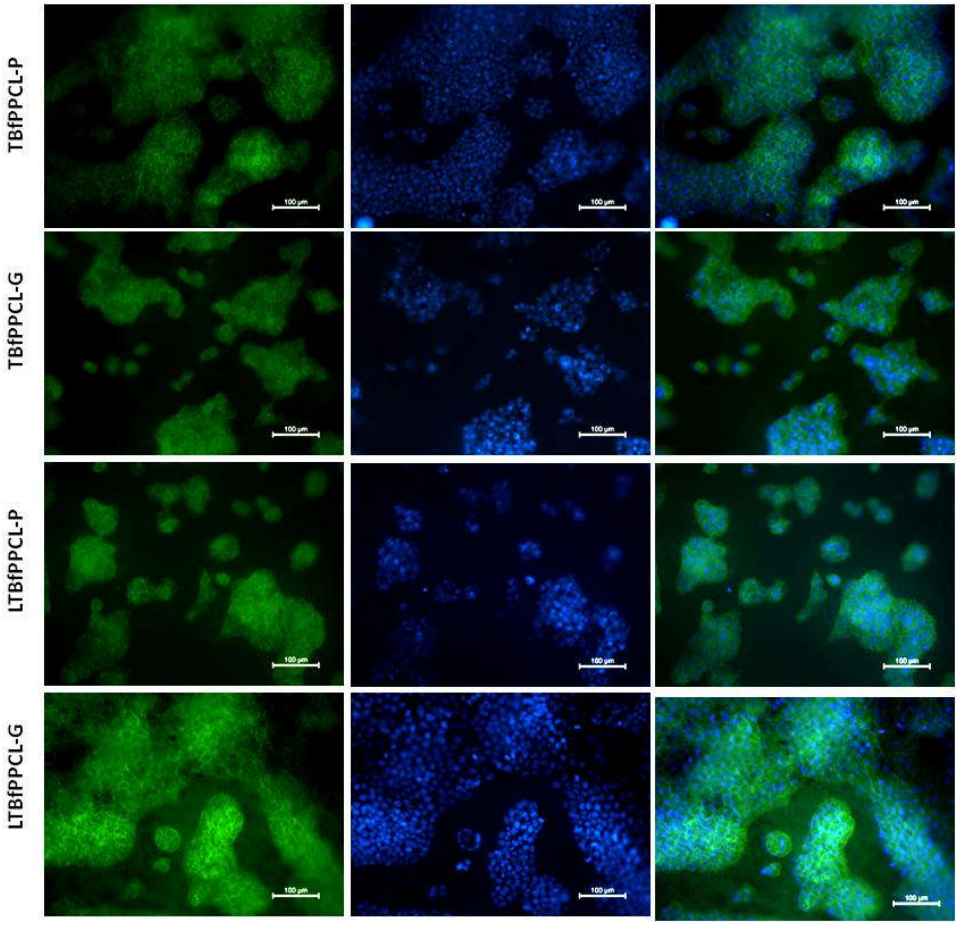

Figure 20. Morphology of HaCaT cells adhered on TBfPPCL-P, TBfPPCL-G, LTBfPPCL-P and LTBfPPCL-G.

Cell viability is an important parameter in the evaluation of biomaterials. The cytotoxic material should also express desired cell adhesion property to confirm that the cells in contact with material is not affecting the normal structural and functional aspects of cells. The viability of HaCaT cells adhered on biofunctionalized TBfPPCL and lyophilized LTBfPPCL mats was assessed by FDA-PI staining. Cells cultured on the surface of materials for 72 h were viable. Viable cells appeared green where as the dead cells appeared red in colour (Figure 21).

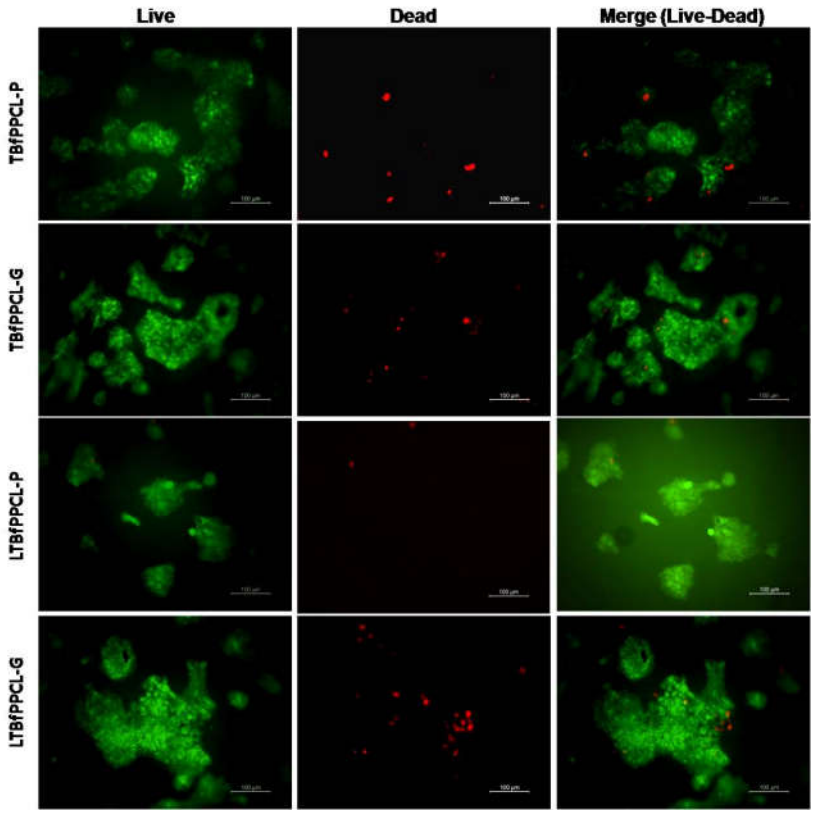

Figure 21. Viability of HaCaT cells on TBfPPCL-P , TBfPPCL-G, LTBfPPCL-P&LTBfPPCL-G. mats.

3.4.4.3 *Test on Extract with MTT Assay of biofunctionalized mats*

Test on extract assay provides potential toxicity due to soluble leachants from biomaterials. The extract of TBfPPCL-P, TBfPPCL-G, LTBfPPCL-P, LTBfPPCL-G were obtained in culture medium with serum at a ratio of 6 cm^2/ml. L-929 cells exposed to various dilutions of extract for 24 h showed normal cell growth and morphology. The metabolic activity of the cells exposed to extracts of materials was assessed by MTT assay. The 100 % extract of TBfPPCL-P, TBfPPCL-G, LTBfPPCL-P and LTBfPPCL-G showed around 70% to 75 % cell activity respectively compared to cells cultured in normal medium (Figure 22 and Figure 23) . However as the dilution increased the cell activity was also increased indicating possible leachants or other components from the mat. The positive control used was phenol with confirmed cytotoxicity. Hence the test was repeated with electrospun mats after additional washing steps to improve the cell activity. The 100% extracts of LBfPPCL-P was around 128 ± 11% and that of LTBfPPCL-G showed cell activity similar to the cell control (Figure 24). This confirmed

the fabrication steps to be sufficient to produce non cytotoxic electrospun mats for wound dressing applications.

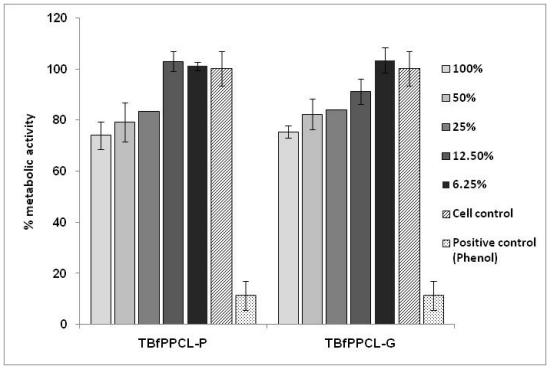

Figure 22. MTT assay of TBfPPCL-P and TBfPPCL-G using L-929

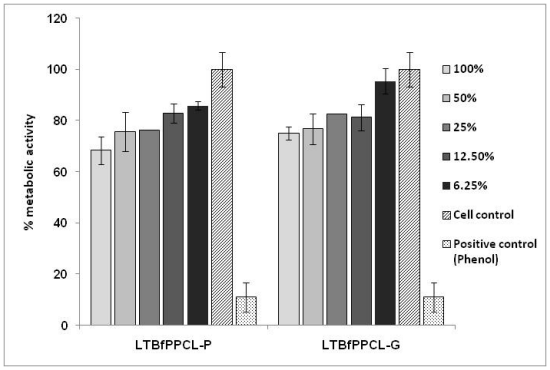

Figure 23. MTT assay of LTBfPPCL-P and LTBfPPCL-G using L-929

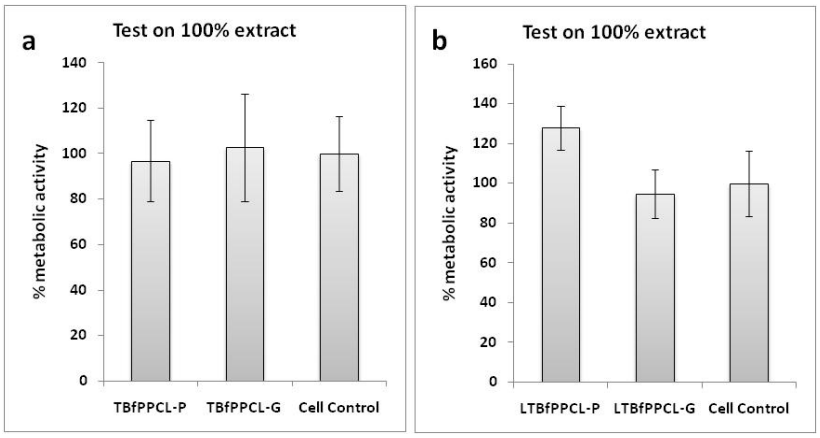

Figure 24. MTT assay of (a)TBfPPCL and (b) LTBfPPCL after additional washing step using L-929.

3.4.4.4 *Cell affinity towards material*

In this study cover glass was used as a control for cell affinity for adhesion. HaCaT cultured on non-treated tissue culture dishes remained non adherent. Keratinocytes seeded on normal tissue culture treated adherent surface and placed TBfPPCL-P, TBfPPCL-G, LTBfPPCL-P and LTBfPPCL-G mats were placed above, the cells could not attach to the mat. The cells were also not able to adhere on the cover glass placed above indicating the affinity of cells towards glass is lesser than that of tissue culture treated plate. When cells cultured on non-adherent dishes and presented with electrospun mats above, the cells did not adhered on the mats. However the cells adhered on the cover glass as it is a good

substrate for cell adhesion compared to non-adherent surface. This clearly indicates the low cell affinity for cell adhesion of TBfPPCL-P, TBfPPCL-G, LTBfPPCL-P and LTBfPPCL-G.

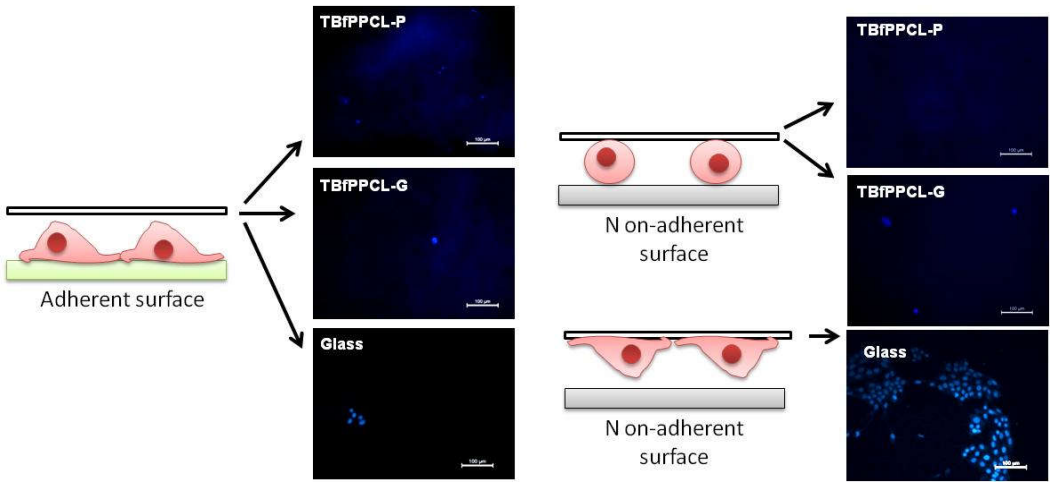

Figure 25. Affinity of Keratinocytes towards TBfPPCL-P, TBfPPCL-G.

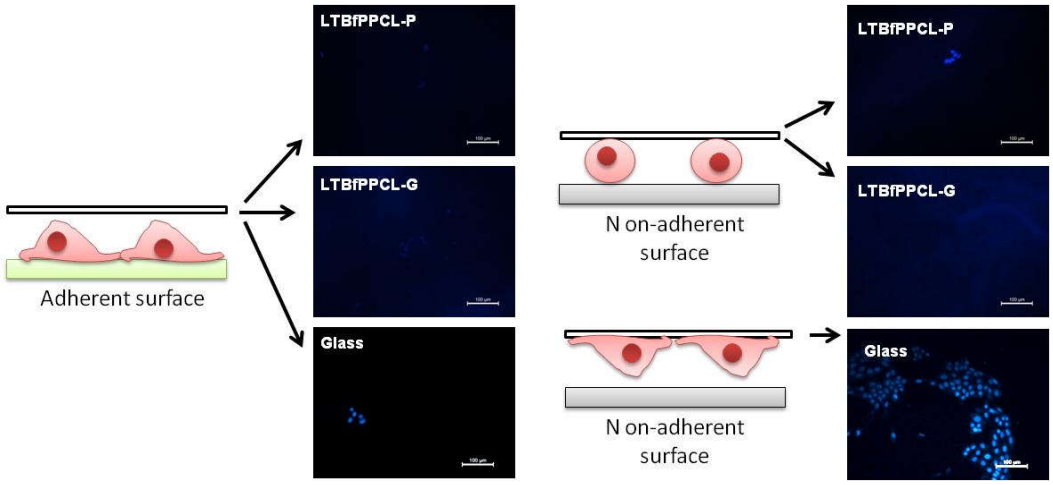

Figure 26. Affinity of Keratinocytes towards LTBfPPCL-P, LTBfPPCL-G.

3.4.4.5 *Hemocompatibility*

Hemocompatibility of the TBfPPCL-P and TBfPPCL-G were evaluated by percentage of hemolysis and percentage change of platelet count. The hemolysis percentage (HP) represents the extent of red blood cells broken by the sample in contact with blood. This experiment shown that hemolysis percentage TPPCL-P, TPPCL-G, LTBfPPCL-P & LTBfPPCL-G were around 0.1% (Figure 26 a).. According to ISO 10993

part 4 hemolysis should be less than 0.1% but according to ASTM F756-00(2000) standard the percentage hemolysis less than 2% is the indication of material with non-hemolysis and good Hemocompatibility [90]. Percentage change of platelet count of PPCL-P, PPCL-G, LTBfPPCL-P & LTBfPPCL-G were in between 20 and 35 %(Figure 27 b). Platelet adhesion and aggression are the important properties which enhances intrinsic blood coagulation [91].

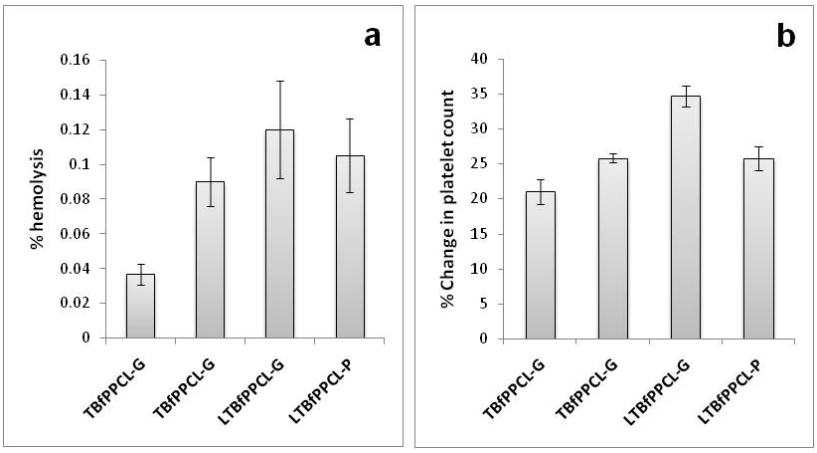

Figure 27. a) Hemolytic nature of TBfPPCL & LTBfPPCL. b) Nature of platelet adhesion of TBfPPCL and LTBfPPCL.

3.4.5 *Ex vivo* Wound model

Ex vivo wound model was proposed to explain the efficiency of TBfPPCL mat to form clot on skin wound. Protein collected from both skin and wound after 20 min of clotting time revealed that the presence of fibrin protein in both skin and mat. Protein band thickness from 10µl samples revealed that the presence of fibrin protein is more in skin than mat. From this data it is expected that. If TBfPPCL mat is used as wound dressing materials it will not cause any further trauma while removing the material.

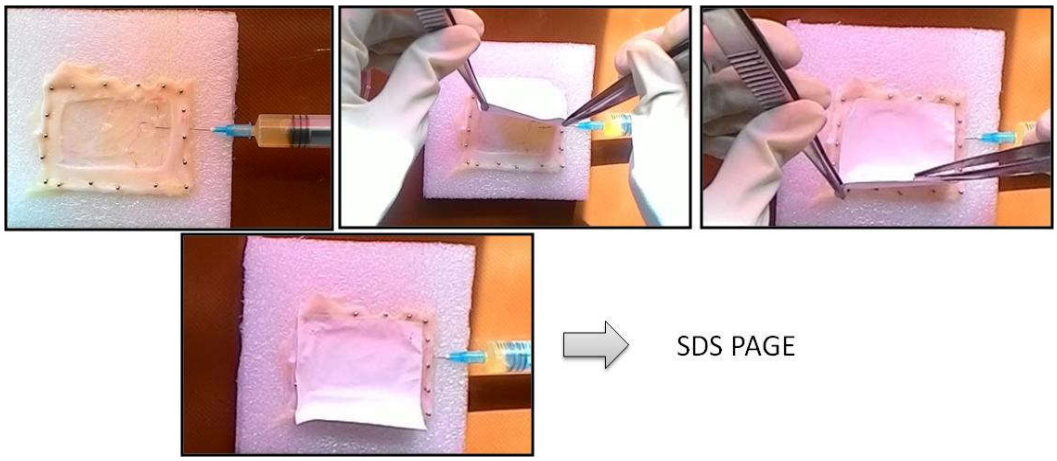

Figure 28. *Ex vivo* wound model using Rabbit skin and TBfPPCL-P mat.

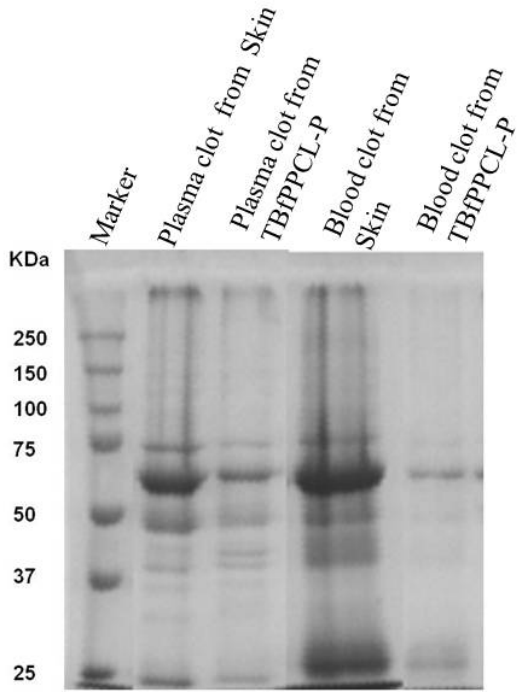

Figure 29. SDS PAGE Analysis to identify the presence of fibrin proteins.

3.5 Biofunctionalized Biodegradable Electrospun mat as a large area tissue sealant.

3.5.1 Biodegradation study of PCL/PLGA Electrospun mat

PLGA is a hydrophilic and highly crystalline polymer with a relatively fast degradation rate and this is the copolymer of PLA (Poly-lactic acid) and PGA (Poly-Glycolic acid). Hydrolytic degradation of PLGA through de-esterification releases its monomeric unit. Once degraded monomeric unit of these polymers are removed by natural enzymatic pathways in body [96]. After 7 days of degradation study only 9% of degradation was observed in PCL-PLGA electrospun mat. Degradation rate increased to 13% at 14th day. According to previously reported data electrospun PCL-PLGA electrospun mat reported to increase the biodegradation rate because PLGA become easily degraded by human body due to the presence of specific enzymes.

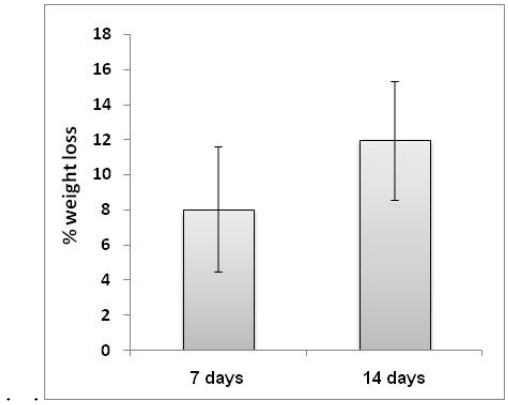

Figure 30. Degradation study of electrospun fibers of PCL-PLGA.

3.5.2 Biofunctionalization of PCL/PLGA mat and Determination of its efficiency

To overcome the limitations of wound dressings applied on bleeding wound the PCL/PLGA electrospun mat was biofunctionalized with thrombin. Thrombin is a key enzyme in coagulation pathway that it converts fibrinogen to fibrin [93]. The efficiency of biofunctionalization of PCL/PLGA mat was assessed by analyzing the formation of fibrin clot when the samples were exposed to human plasma. Protein estimation done after 20 min of clotting time revealed that it is suitable for wound healing application with hemostatic property as same as that of PPCL-P and PPCL-G because the Concentration of fibrin protein obtained from PPCL-P, PPCL-G and PCL/PLGA mat were almost similar (Figure:30)

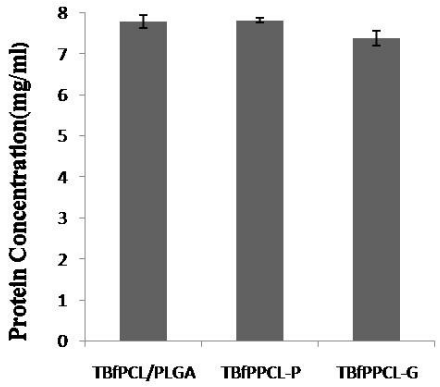

Figure 31.Comparison of Fibrin protein Concentration after clot formation on PCL-PLGA, TBfPPCL-P, TBfPPCL-G mats.

3.6 Device models using PPCL electrospun mat

3.6.1 Modification of Electrospun mat for fluid absorption

3.6.1.1 *Fluid absorption of SAP*

The absorption ability of super absorbent sodium polyacrylate was analyzed using blood and deionized water. 100 mg SAP could absorbe approximately 1.5 ml blood. The weight of SAP required for absorbing same quantity of water was only 10 mg. According to previous report sodium polyacrylate have good water absorption ability of 500-1000 times [97].

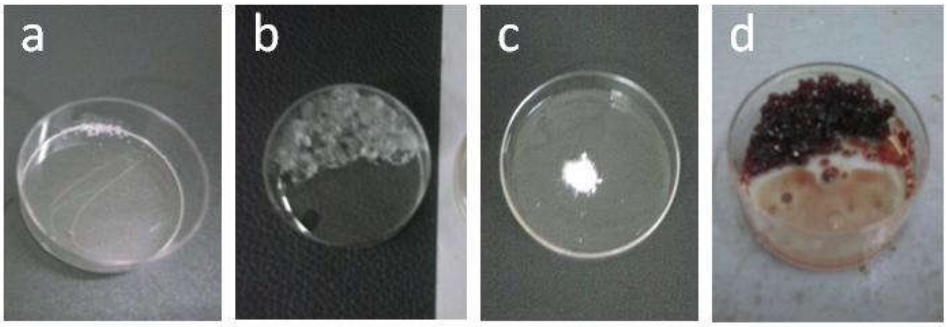

Figure 32. a&c) SAP before fluid absorption. b)SAP after absorption of water. d)SAP after absorption of blood.

3.6.1.2 *Fluid absorption by SA-Band*

Moisture and nutrients are essential elements to provide proper wound healing environment and wound exudates plays an important role. However, highly exuding wounds can affect the healing process and the surrounding tissues. Hence management of exuding wound is taken into account while considering variety of other factors. Exudate management dressings are recommended in such cases [98]. In this study PCL mat was modified by using sodium polyacrylate and gauze to gain the property of fluid absorption. This modified material has efficiency to absorb water immediately and blood within 20 to 25 minutes. Swelling of material was observed as a result of fluid absorption. One of the commercially available wound dressing materials consisting of sodium polyacrylate is 3M™ Tegaderm™ Superabsorber. Such material expresses high exudates absorption efficiency in chronic and acute exuding wounds. A super absorbent bandage (SA-Band) is proposed here which was developed using electrospun PPCL mats. The SA-Band showed high water absorbing property as the same time expressed whole blood absorbing ability. The bandage was assembled in the form of a wound dressing device.

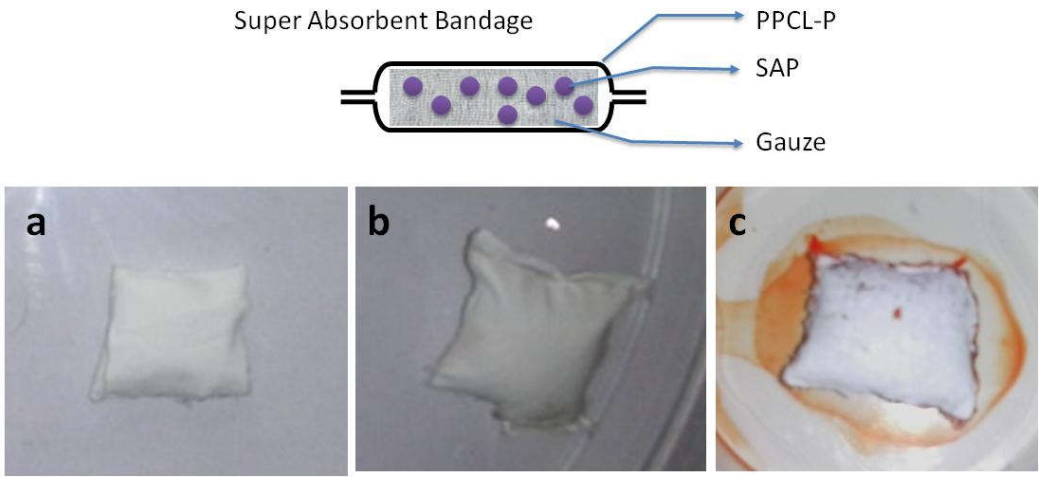

Figure 33.a)Fresh SA-Band, b) Swelling of SA-Band after absorption of water, c)Swelling of SA-B after absorption of blood.

Chapter 4

SUMMARY AND CONCLUSIONS

Skin is the largest organ of the body which gives protection from harmful biological and chemical agents. It also helps to regulate body temperature, and permits the sensation of touch, heat and cold. Skin wound break down all these protective functions because of loss of continuity of epithelium with or without underlying connective tissue. Hemostasis is the normal mechanism in body, functions to heal wounds by synthesizing natural fibrin clot as wound healing matrix. But in case of large area wounds this natural matrix doesn't provide complete protection in such situations different types of wound dressing materials can use to heal wound completely.

Porous electrospun mat was fabricated by using THF:DMSO as solvent. Two types of electrospun mats were fabricated by changing the collector type. Physicochemical and biological analysis revealed that it is suitable for wound management. Thrombin is a natural enzyme present in circulatory system, its main function to form fibrin clot from protein fibrinogen. In this study thrombin biofunctionalized porous Polycaprolactone was fabricated as hemostatic agent to prevent excess blood loss from large area bleeding skin wounds. Biofunctionalization efficiency of mat was analyzed by protein estimation; protein characterization done by SDS-PAGE analysis confirmed the presence of fibrin. Biological evaluation of biofunctionalized mat confirmed that it is biocompatible. Instant clotting together with fast biodegradability will help in large area tissue defects such as burns. PLGA is a biodegradable polymer was used to increases the biodegradation rate of electrospun mat mat. PCL/PLGA electrospun mat was fabricated and, its degradation study gave 9% degradation on 7^{th} and 13% degradation on 14^{th} day. Wound exudates produce as a normal wound healing mechanism to prevent drying out and to improve healing of wounds. High amount of exudates production in chronic wounds prolong the inflammatory phase because inflammatory substances present in the exudates breakdown the cell supporting extracellular matrix, in such situation exudates management provide favorable environment for wound healing. Sodium polyacrylate is a super absorbent polymer was used to fabricate device model for highly exuding wounds and evaluation of above device showed that it is suitable to absorb high amount of fluids.

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