NANOFIBROUS MAT FOR TISSUE ENGINEERING, WOUND DRESSING AND DERMAL RECONSTITUTION

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NATIONAL UNIVERSITY OF SINGAPORE (NUS)

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Summary

The current design for a tissue engineering (TE) skin substitute is that of a biodegradable scaffold through which fibroblasts can migrate and populate. This artificial 'dermal layer' needs to 'take' (adhere and integrate) to the wound, which is not always successful for the current artificial dermal analogues available (e.g. Integra®, Dermagraft® or TransCyte®). The high cost of these artificial dermal analogues also makes its application prohibitive both to surgeons and patients, and in certain cases, ethical issues may be involved too.

Here, we propose a cost-effective composite consisting of a nanofibrous scaffold directly electrospun onto a Tegaderm™ wound dressing (TG-NF construct) for dermal wound healing. Cell culture is performed on both sides of the nanofibrous scaffold and tested for fibroblast integration and proliferation. It is hoped that these studies will result in a fibroblast populated three-dimensional dermal analogue that is feasible for layered applications to build up thickness of dermis prior to re-epithelialisation. The extent of injuries looked into largely refer to full or partial thickness injuries to the dermal tissues such as burn and chronic wounds.

Results obtained in this study suggest that both the TG-NF construct and dual-sided fibroblasts populated nanofiber construct, achieved significant cell adhesion, growth and infiltration. This is a successful first step for the nanofiber construct in establishing itself as a suitable three-dimensional scaffold for autogenous fibroblasts population, and provides great potential in the treatment of dermal wounds through layered application.

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Chapter 1: Introduction

The skin is the largest organ in the human body, covering the entire external surface and forming about 8% of the total body mass. The surface area of skin varies with height and weight. For an individual 1.8m tall and weighing 90kg, it covers about 2.2m^2. Thickness of skin varies from 1.5 – 4.0 mm, depending on skin maturity (ageing) and body region.

The skin forms a self-renewing and self-repairing interface between the body and the environment. It provides an effective barrier against microbial invasion, and has properties that can protect against mechanical, chemical, osmotic, thermal and photo damage. It is capable of adsorption and excretion, and is selectively permeable to various chemical substances. [1]

Skin also has good frictional properties, assisting locomotion and manipulation by its texture. Being elastic, it can be stretched and compressed within limits. The general state of health is commonly reflected by the appearance and condition of the skin, with the earliest signs of many systematic disorders being detected by inspection. Examination of skin is therefore important in diagnosing more than just skin diseases. [1, 2, 3]

Skin is a relatively soft tissue and must be able to withstand large shear stresses. Trauma to the skin can be caused by many factors such as heat, chemicals, electricity, ultraviolet radiation or nuclear energy, and can result in several degrees of skin damage. The least damaging traumas tend to wound only the epithelium, which is the most superficial layer of skin. Wounded epithelium generally is healed by the body via re-epithelialization and

does not require skin grafting. More serious trauma can lead to partial or complete damage to both dermal and subdermal tissues. [4] Wounds that extend partially through the dermis are capable of regeneration; the dermis provides a source of cells for its own reconstitution, while deep skin appendages such as hair follicles and sweat glands provide sources of epidermal cells to recreate the epidermis. Unfortunately, the body cannot heal deep dermal injuries adequately. In these cases, such as full thickness burns or deep ulcers, there are no remaining sources of cells for regeneration. [5]

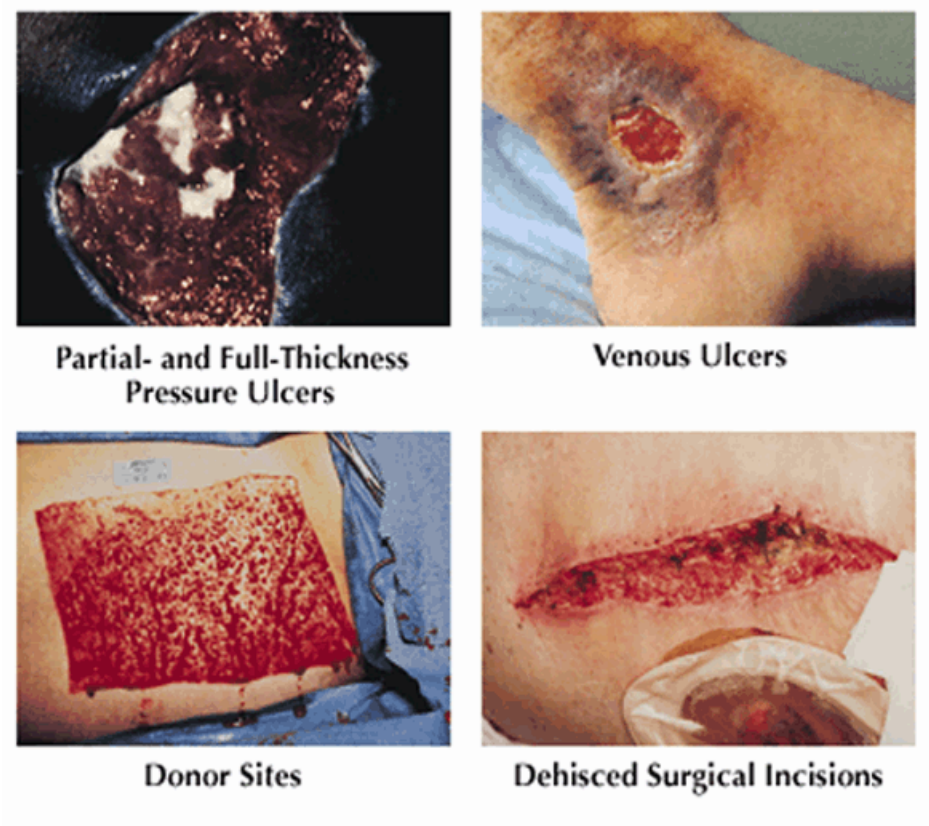

Figure 1.1: Partial and full thickness burn wound

(http://www.jnjgateway.com)

According to worldwide statistics, an astonishing annual amount of US$5 billion is required for burn wound care. In the United Kingdom (UK), £600 million per year is expended for the treatment of chronic leg ulceration. In the United States (USA), US$9 billion is spent per annum on wound care products. The average cost for wound healing ranges from US$27,000 for a pressure ulcer, to US$36,000 for a diabetic wound. In Singapore, an estimated annual expenditure of US$180 million goes into the treatment of chronic wounds. The UK and US statistics are now 10 years old, but with increasing cases of burn injuries arising from fire, accidents, terrorist attacks and an aging population (for chronic wounds), the numbers could now be much higher. These statistics show a potentially huge market for wound care products, and stress the need to lower the cost for dermal rehabilitation so that it can be made as easily available to the public as possible.

1.1Scope of Work

The current design for a tissue engineering (TE) skin substitute is that of a biodegradable scaffold through which fibroblasts can migrate and populate. This artificial 'dermal layer' needs to 'take' (adhere and integrate) to the wound, which is not always successful for the current artificial dermal analogues available (e.g. Integra®, Dermagraft® or TransCyte®). The high cost of these artificial dermal analogues also makes its application prohibitive both to surgeons and patients, and in specific cases, ethical issues may be involved too.

1.2 Objectives

In this study, cell culture work is performed on both sides of individual PCL/gelatin nanofiber construct and also electrospun nanofibrous scaffold on Tegaderm^{TM} wound dressing, which will be termed as the Tegaderm-Nanofiber (TG-NF) construct. A new technique is being put forth, where fibroblasts are cultured on both sides of a relatively thin construct of nanofibrous scaffold. This would increase the capacity of cells to be loaded into the scaffold structure and also engage cellular penetration from both sides of the scaffold instead of the usual convention of using one side of the nanofibrous scaffold. Our objectives in this study are to:

- Investigate the feasibility of using the electrospun TG-NF construct as a cost-effective scaffold for fibroblast integration and proliferation to establish a fibroblast populated dermal analogue,
- Examine the possibility of dual-sided cellular growth on a nanofiber scaffold so as to establish a three dimensional fibroblast populated dermal analogue, and through which establish the feasibility of using the conceptualized autologous layered dermal reconstitution (ALDR) approach for layered applications of this TG-NF construct to build up thickness of dermis prior to re-epithelialisation

The extent of injuries looked into largely refer to full or partial thickness injuries to the dermal tissues such as burns and chronic wounds.

Chapter 2: Literature Review

2.1 Nanofibrous Scaffold Fabrication Technique – Electrospinning

Electrospinning has been known for over 60 years in the textile industry for manufacturing non-woven fiber fabrics. In recent years, there has been an increasing interest in exploiting this technology in the emerging area of nanobioengineering, which focuses mainly on using nanometer scale fibers for biomedical applications. This is especially so in fabrication of the nanofibrous scaffold for tissue engineering (TE). This technique produces non-woven membranes with individual fiber diameters ranging from 50 to 800nm. The fibers form a large interconnected porous network that is ideal for drug, gene as well as cell delivery.

In electrospinning, many factors can be manipulated in order to generate scaffolds of different geometries and with different structural properties. These include electrospinning parameters such as the electric field strength, the distance of electric field, the length and radius of the spinneret, the solution flow rate, and solution parameters such as concentration, viscosity, ionic strength and conductivity. [7]

There is an increasing interest towards employing electrospinning for scaffold fabrication because the mechanical, biological and kinetic properties of the scaffold are easily manipulated by altering the polymer solution composition and processing parameters. Another advantage of using the electrospinning process is the ability to produce a nonwoven, nanometer scale fibrous structure, which has morphological and architectural features similar to that of the natural ECM, which comprises three dimensional network

of nanoscaled fibrous protein structures embedded in glycosaminoglycan (GAG) hydrogel. [8] Additionally, the scaffold structure changes dynamically over time as the polymer nanofiber degrade, allowing the seeded cells to recreate and consolidate their own ECM. [9] In addition to the electrospinning of polymer materials, there are also approaches to electrospin natural biomaterials like collagen, fibrinogen and gelatin. [10 – 14]

Research in recent years has also established the widespread application of electrospun nanofibers in tissue engineering of bone [15], blood vessels [16], cartilage [17], peripheral nerve system [18], ligament [19], skeletal muscles [20], cardiac tissues [21] and skin [22].

The nanofibrous scaffolds we present in this paper are electrospun from co-polymer poly (ε-caprolactone) (PCL)/gelatin (Type A) of 10% wt, with 2,2,2-trifluoroethanol (TFE) as the solvent. PCL is bioresorbable, biocompatible and has been studied as a wound dressing material as far back as the 1970s. Extensive research had been conducted on biocompatibility and efficacy, both *in vitro* and *in vivo*, resulting in FDA approval of a number of medical and drug delivery devices that are composed of PCL. At present, PCL is being regarded as a soft and hard tissue compatible bioresorbable material [23]. Gelatin is a natural biopolymer derived from collagen by controlled hydrolysis. Because of its many merits, such as its biological origin, biodegradability, biocompatibility, and commercial availability at relatively low cost, gelatin has been widely used in the pharmaceutical and medical fields. Preliminary studies have shown that the co-polymer

of PCL/gelatin provides a compromised solution for overcoming the shortcomings of natural and synthetic polymers, resulting in a new biomaterial with good biocompatibility and improved mechanical, physical and chemical properties. [14]

2.2 Basic Skin Anatomy

Skin is one of the few organs of the body that are capable of regeneration. The two main layers of the skin include the epidermis, composed of stratified squamous epithelium, and the dermis, made up of dense connective tissue and fibroblasts in an extracellular matrix (ECM). The epidermis constantly proliferates and replaces itself while the dermis is rich in connective tissue that provides high tensile and elastic strength. (Figure 2.1)

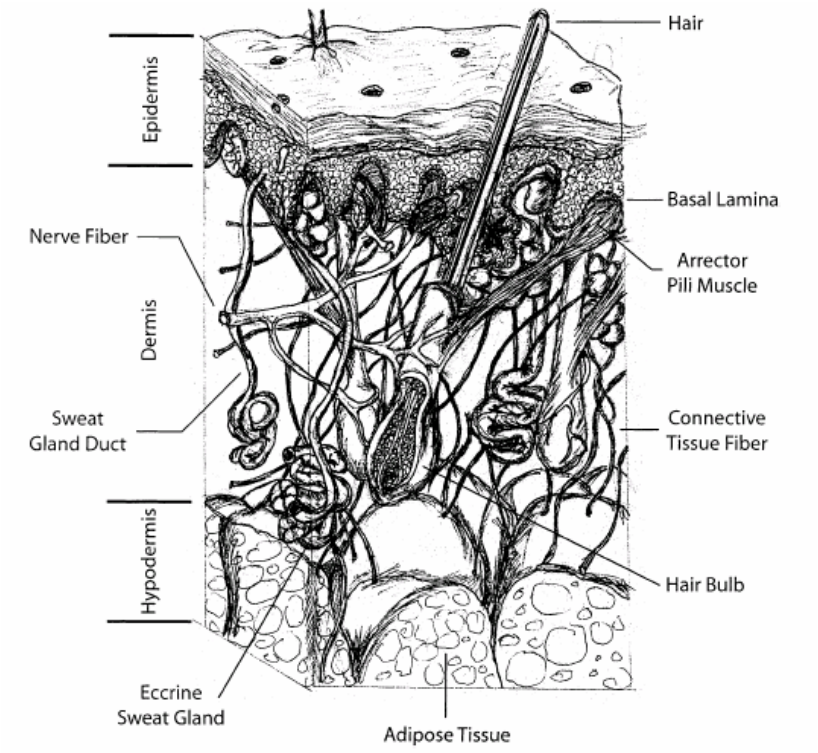

Figure 2.1: A pen drawing of complex structure of skin

(M.H. Ross et al –History: A text and Altas, Williams and Wilkins, Baltimore, 1995)

The hypodermis comprises a looser connective tissue that lies beneath the dermis. The skin possesses sensory receptors, hair follicles for the production and growth of hair, and sweat glands which primarily regulate body temperature. [4]

2.2.1 The Epidermis

The skin is a physical barrier between the body and the external environment. The outermost layer of skin, the epidermis, must therefore be tough and impermeable to toxic substances or harmful organisms. It also controls the loss of water from the body to the relatively drier external environment. The epidermis is home to epithelial cells, keratinocytes as well as melanocytes (which gives skin its color and protection against ultraviolet rays). Proliferating cells in the basal layer of the epidermis anchor the epidermis to the dermis and replenish epithelial cells lost through normal exfoliation of the skin surface. The most superficial keratinocytes in the epidermis form the dead outermost structure that, by its cornified nature, provides for mechanical barrier properties of the skin. [6]

2.2.2 The Dermis

The dermis underlies the epidermis. It provides physical strength and flexibility to skin as well as the matrix that supports the extensive vasculature, lymphatic system, and nerve bundles. The dermis is relatively accellular, composed predominantly of an ECM of interwoven collagen fibrils. Fibroblasts, the major cell type of the dermis, produce and maintain most of the ECM. Endothelial cells line the blood vessels and play a critical role

in the skin immune system by controlling the extravasation of leukocytes. A network of nerve fibers extends throughout the dermis, serving a sensory role in the skin. These nerve fibers also secrete neuropeptides that influence immune and inflammatory responses in skin through their effect on endothelial cells, leukocytes and keratinocytes. [6]

2.3 Skin or Cutaneous Wound Healing

Since skin forms a protective barrier around the body, damage to the skin poses several immediate threats including rapid, severe dehydration and infection. [4] In burn wounds, these are life threatening injuries which require immediate care followed by careful evaluation and appropriate wound management. The severity of burns (Figure 2.2) will also affect the degree of scarring the victim experiences.

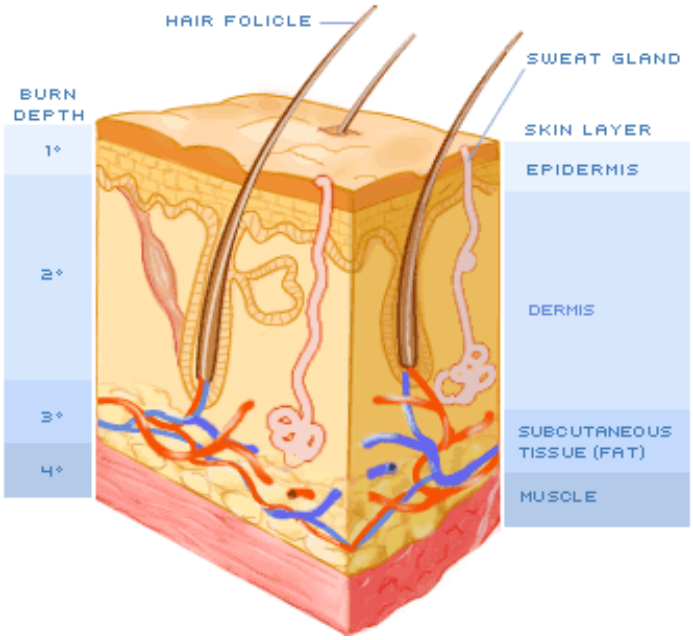

Figure 2.2: Skin layer and burn depth diagram

(http://www.skinhealing.com)

To prevent these, the standard treatment is an autograft, where a thin section of skin (including both the epidermis and partial thickness dermis) is removed from an unaffected part of the body and grafted onto the wound. The grafted skin will attach itself to the underlying tissue and effectively close the wound. A graft is successful when new blood vessels and tissues from the base integrate with the graft (take).

However, skin grafts do not always take because of complications such as infection (most common cause of graft failure) or shearing (pressure causing the graft to detach from skin). When burns are widespread, there may be insufficient healthy skin available to graft onto the burn area. Healing by secondary intention over a prolonged period

commonly result in poor scarring, leading to cosmetic and functional deformities. Restoration of skin loss using TE has overcome some of these problems.

2.4 Synthetic Dermal Analogues

The lack of sufficient donor sites on many burn and chronic wound patients and the generation of a donor defect by skin grafting have prompted the search for more widely available skin substitute. As a result, certain skin alternatives have been developed over the years and have obtained approval from the U.S Food and Drug Administration (FDA) for use in skin reconstructive procedures.

2.4.1 Integra® Dermal Regeneration Template

Integra® Dermal Regeneration Template (Integra Life Sciences, Plainsboro, NJ, USA) was approved by the FDA in 1996 for use in treatment of life threatening full or deep partial-thickness dermal injuries where sufficient autograft is not readily available at the time of excision or is undesirable due to the physiologic condition of the patient.

Integra® is made of a bi-laminate membrane consisting of a bovine collagen-based dermal analogue and a temporary epidermal substitute layer of silicone. The dermal replacement layer of Integra® consists of a porous matrix of fibers of bovine type I collagen that is crosslinked with chondrotin-6-sulfate, and glycosaminoglycan (GAG) extracted from shark cartilage. The porous matrix is designed to act as a template for infiltration of the patient's fibroblasts, macrophages, lymphocytes, and capillaries. The outer silicone layer of Integra serves as a temporary epidermis and allows for limited fluid flux, protection from microbial invasion, and prevention of wound dehydration.

Figure 2.3: Integra® Dermal Regeneration Template

(http://www.integra-ls.com/bus-skin_product.shtml)

Integra® is placed on the excised wound for approximately 2 to 3 weeks, and during that time, the dermal component incorporates itself into the patient's wound producing a neodermis. After the neodermis has been formed, the silicone layer is removed and a thin epidermal autograft of 0.005 inch may be applied with minimal donor site deformity. During the period between Integra® placement and epidermal autograft laying, the Integra® construct must be protected from mechanical dislodgement and observed daily for signs and symptoms of infection. A histologic evaluation of Integra® by Stern *et al.* [26] was conducted in 1990. 336 serial biopsies obtained from 131 patients in periods ranging from 7 days to 2 years after application of Integra®, showed restoration of an intact dermis with definitive closure of wound and minimal scarring. [24, 25]

However, the disadvantage of Integra® application is the difficulty in obtaining an ultra-thin epidermal autograft. Its thickness in dimension also causes difficulties for Integra® to 'take' to the wound site. In addition, the small size of the Integra and high cost involved in its production makes it non cost effective and not readily affordable to the general public.

2.4.2 Dermagraft® - Human Fibroblast-Derived Dermal Substitute

Dermagraft® (Advanced Tissue Sciences, La Jolla, CA, USA) is a cryopreserved human allograft fibroblast-derived dermal substitute comprising of fibroblasts, ECM and a bioabsorbable scaffold.

Figure 2.4: Dermagraft® – Human Fibroblast-Derived Dermal Substitute

(http://www.advancedbiohealing.com)

Human fibroblast cells are expanded and seeded onto a bioabsorbable scaffold under aseptic conditions. The cells are tested throughout the process for bacteria, viruses, fungi, and mycoplasma. The cells attach to the scaffold and multiply to fill the spaces within the scaffold during a 2-3 week period. They continue to divide and grow, secreting human growth factors, cytokines, ECM proteins and glycosaminoglycans. The end result is a cryopreserved three-dimensional human dermal substitute containing metabolically active living cells. [27]

A pilot study evaluated healing in 50 patients with diabetic foot ulcers treated with Dermagraft®. Ulcer healing was determined by the percentage of wound that achieved complete or 50% wound closure, time to closure, and volume and area measurements. Patients who received Dermagraft®, applied weekly for eight consecutive weeks, healed significantly faster than patients treated with traditional wound closure methods. In a larger controlled study of diabetic foot ulcers on 281 patients, it was also concluded that the Dermagraft® healing rate was higher compared to the control group. In addition, Dermagraft® appeared to delay ulcer recurrence in this study. [28]

However, Dermagraft® cannot be used in ulcers that have signs of clinical infection or sinus tracts. Dermagraft® has also not been studied in wounds that extend to the tendon, muscle, joint capsules or bone. [27]

2.4.3 TransCyte® – Human Fibroblast-Derived Temporary Skin Substitute

TransCyte® (Advanced Tissue Sciences, La Jolla, CA, USA) consists of a polymer membrane and neonatal human fibroblast cells cultured under aseptic conditions in vitro on a porcine collagen coated nylon mesh. It acts as a temporary wound covering for surgically excised full-thickness and partial-thickness wounds, to protect the wound from environmental insults. In addition, the membrane is semi-permeable, thus allowing fluid and gaseous exchange.

Figure 2.5: TransCyte® – Human Fibroblast Derived Temporary Skin Substitute

(http://www.advancedbiohealing.com)

Before incorporating the cells, this nylon mesh is coated with porcine dermal collagen and bonded to a polymer membrane (silicone). This membrane provides a transparent synthetic epidermis when the product is applied to the wound. As fibroblasts proliferate within the nylon mesh during the manufacturing process, they secrete human dermal

collagen, matrix proteins and growth factors. Following freezing, no cellular metabolic activity remains. However, the tissue matrix and bound growth factors are left intact. The human fibroblast-derived temporary skin substitute provides a temporary protective barrier. It is transparent and allows direct visual monitoring of the wound bed. [29]

Noordenbos *et al.* [30] conducted a randomized comparison study of silver sulfadiazine and TransCyte® using paired burn wound sites on 14 patients and a non-comparison evaluation on 18 patients. The investigation showed that burn wounds treated with TransCyte® healed more quickly than burn wounds treated with silver sulfadiazine (mean = 11.4 days to 90% epithelialization vs. 18.14 days). No infection occurred on the 32 burn wounds treated with TransCyte®. In addition, the burn wound site evaluations completed at 3, 6 and 12 months revealed less hypertrophic scarring on TransCyte® treated wounds. [30]

However, TransCyte® cannot be applied to patients who are sensitive to porcine dermal collagen. TransCyte® may also contain small traces of animal proteins due to exposure in the manufacturing process, and similarly in the pre-coating of the nylon mesh with porcine dermal collagen. The exposure of animal proteins to patients has been reduced by covering the nylon mesh with naturally secreted dermal protein. [29] Ethical issues pose a problem here too, as patients of certain religious faiths may not accept the porcine-derived TransCyte® and the unknown source of human fibroblast cells used.

TransCyte® is not suitable for prolonged application because it may result in immunological rejection by the patient. It has also not been established for application in burns of the head or hands, or in surgically excised full-thickness and deep partial-thickness wounds prior to autografting. The nylon mesh used in TransCyte® is also not biodegradable. [24] In clinical trials, the only complication was that of fluid accumulation in the wound sites.

2.5 Tegaderm™ Wound Dressing

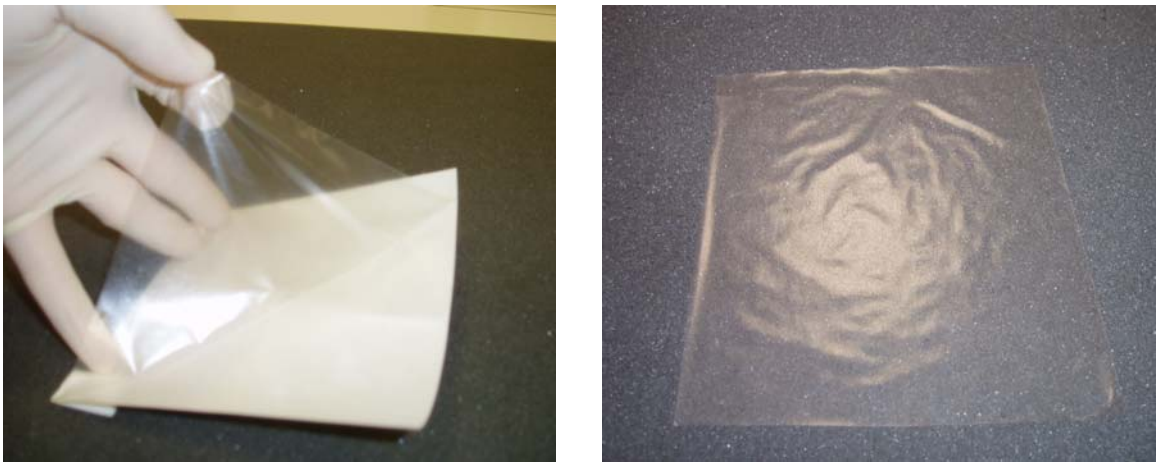

Figure 2.6: Tegaderm™ wound dressing, 3M (without acrylic adhesive)

Tegaderm™, a synthetic gas permeable membrane, is currently used as a common wound dressing (Figure 2.6). It is inexpensive, and earlier studies have shown it to be a reliable carrier for cultured epithelial allograft (CEA) in burns resurfacing. [32] It consists of a thin polyurethane membrane coated with a layer of an acrylic adhesive. The dressing functions such that it is selectively permeable to both water vapor and oxygen, and yet impermeable to microorganisms. Once placed in position, it provides an effective barrier to most forms of external contamination, and is still capable of maintaining a moist environment at the surface of the wound by reducing water vapor loss from the exposed tissue. Under such conditions in shallow wounds, scab formation is prevented and epidermal regeneration takes place at a faster rate as compared with regeneration in wounds treated with traditional dry dressings.

The Tegaderm™ wound dressing can be used in the treatment of minor burns, pressure areas, donor sites, post-operative wounds, and a variety of minor injuries including abrasions and lacerations. It can also be used to cover and protect wounds, to maintain a moist environment for wound healing, as a secondary (cover) dressing, and as a protective cover over at-risk skin. It is not recommended that the material be applied over deep cavity wounds, third degree burns or wounds that show evidence of clinical infections.

From previous works [34, 35], we have also concluded that Tegaderm™ is cheap, easily available, supports cell growth and can be used for TE of skin.

In this current study, cell culture work is performed on both sides of individual PCL/gelatin nanofiber construct and also electrospun nanofiberous scaffold on Tegaderm™ wound dressing, which will be termed as the Tegaderm-Nanofiber (TG-NF) construct. A new technique is being put forth, where fibroblasts are cultured on both sides of a relatively thin construct of nanofibrous scaffold. This would increase the capacity of cells to be loaded into the scaffold structure and also engage cellular penetration from both sides of the scaffold instead of the usual convention of using one side of the nanofibrous scaffold. For the TG-NF construct, the nanofibrous scaffold allows fibroblast cells to have a structure for population and growth with the Tegaderm™ dressing taking on the role of a synthetic epidermis, providing a protective barrier from external infections and maintaining a moist environment for dermal regeneration to take place.

Chapter 3: Materials and Experimental Methodology

3.1 Materials

The polymers gelatin Type A (approx. 300 Bloom, Sigma, St. Louis, MO) from porcine skin in powder form and poly (ε-caprolactone) (PCL) with an average molecular weight of 80,000 (80 kDa), were used together with 2,2,2-trifluoroethanol (TFE) as solvent. The polymers and solvent were of analytical grade and used as received from Fisher Chemicals (Leicestershire, UK). Human dermal fibroblast (HDF) cells were obtained from an 8-month-old Chinese infant (CellResearch Corporation). All culture media and reagents were purchased from Research Biolabs (Sigma, St Louis, MO, USA). CellTiter 96® AQ_{ueous} one solution reagent was purchased from Promega (Madison, WI, USA). CMFDA fluorescent CellTracker probe was purchased from Molecular Probe (Eugene, OR, USA).

3.2 Fabrication of PCL/gelatin scaffolds and TG-NF constructs

A polymer blend solution of PCL/gelatin/TFE was prepared by mixing 10%w/v PCL/TFE and 10%w/v gelatin/TFE in 50:50 portions under gentle stirring to be used for the nanofibrous scaffold preparation. The nanofibrous scaffolds were subsequently electrospun using the electrospinning setup (Fig. 3.1). The TG-NF constructs were fabricated in similar fashion, but with the PCL/gelatin nanofibers being electrospun directly onto the Tegaderm™ wound dressing (Fig. 3.2).

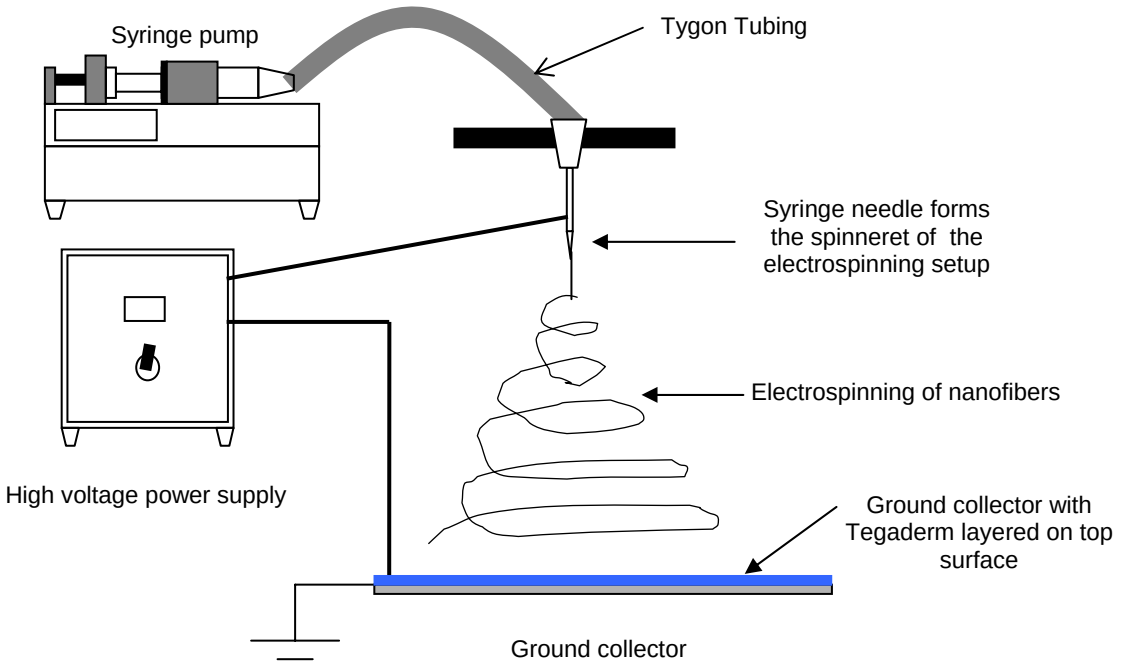

Figure 3.1: Schematic diagram for electrospinning apparatus

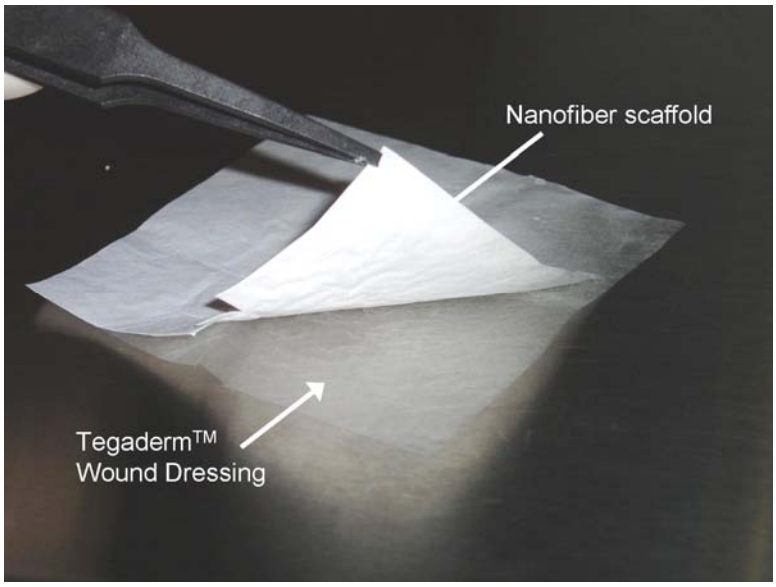

Figure 3.2: Tegaderm-Nanofiber (TG-NF) construct

For the process of electrospinning, the polymer solution was placed in a 10ml plastic syringe fitted with a needle with tip diameter of 0.4mm. Electrospinning voltage was applied between the needle and the grounded collector at 10.5kV using a high voltage power supply (Gamma High-Voltage Research, FL, USA). Above a critical voltage, a polymer jet was ejected from the needle tip towards a grounded collector which was laid with either a Tegaderm^{TM} wound dressing or aluminium foil. The needle is located at a distance of 150mm from the gound collector. A syringe pump was used to feed the polymer solution to the needle tip at a feed rate of 0.7 ml/h. The constructs were dried under vacuum at room temperature.

The thickness of the PCL/gelatin nanofibrous scaffolds was measured with the aid of a micrometer (Mitutoyo, Japan) and its apparent density, bulk density and porosity were estimated through the following equation [34]:

PCL/gelatin bulk density (g/cm^3)

$$= \frac{Mass\_of\_PCL(g) + Mass\_of\_gelatin(g)}{Volume\_of\_PCL/gelatin\_solution(cm^3)} \quad \ldots (3.1)$$

PCL/gelatin nanofibrous scaffold apparent density (g/cm^3)

$$= \frac{Mass\_of\_scaffold(g)}{Scaffold\_thickness(cm) \times Scaffold\_area(cm^2)} \quad \ldots (3.2)$$

PCL/gelatin nanofibrous scaffold porosity (%)

$$= \left(1 - \frac{Scaffold\_apparent\_density(g/cm^3)}{Bulk\_density\_of\_PCL/gelatin(g/cm^3)}\right) \times 100\% \quad \ldots (3.3)$$

3.3 Characterization of PCL/gelatin scaffolds and TG-NF constructs

For characterization, the constructs were sputter coated with gold (JEOL, JFC-1200 Fine Coater, Japan). Surface morphology of the constructs was obtained using the Field Emission Scanning Electron Microscopy (JEOL, JSM-5600, Japan) at an accelerating voltage of 15 kV. All the prepared constructs were sterilized with ultraviolet (UV) light for 2 hours and prewet with 70% ethanol for up to 24 hours. This wetting process is important for hydrophobic polymers like PCL, as it aids in overcoming the hindered entry of water into the air-filled pores within the scaffolds [16]. Subsequently, the scaffolds and constructs were soaked in Dulbecco's modified Eagle's medium (DMEM; Sigma, St Louis, MO USA) with 10% fetal bovine serum (FBS; Sigma, St Louis, MO, USA) overnight to ensure protein adsorption which would facilitate cell attachment.

3.4 In vitro culture of HDFs

HDFs were plated as a monolayer and cultured to confluence in DMEM containing 10% FBS and 1% antibiotic solution (penicillin-streptomycin; Sigma, St Louis, MO, USA). Media was replaced every three days and the cultures were maintained in a humidified incubator at 37^{o}C with 5% CO_2. Once the HDFs reached 70% confluence, they were

trypsinized and passaged at 1:3 ratios. Second to fifth passages of HDFs were used in this study.

3.5 HDFs seeding onto PCL/gelatin scaffolds and TG-NF constructs

HDFs were detached with 1X trypsin and the number of cells was counted with a hemocytometer. The resulting cells in suspension were then seeded onto the TG-NF constructs and incubated for 7 days, with PCL/gelatin nanofiber scaffolds as control. For a separate set of study, HDF cells were also seeded separately onto the PCL/gelatin nanofiber scaffolds as well as tissue culture plastic (TCPS) of the 24-well plates, which was to serve as a negative control. The HDF cells were first seeded on the top surface of the PCL/gelatin nanofiber scaffold and incubated for 7 days. The scaffolds were then flipped over, and incubated for another 7 days with new HDF cells seeded again on the bottom surface. Culture media used was changed every 3 days.

3.6 Field emission scanning electron microscopy (FESEM)

FESEM micrographs of the TG-NF constructs and PCL/gelatin nanofiber scaffolds were taken when HDFs attached and proliferated on the different substrates 3, 5 and 7 days after seeding. Briefly, the substrates were harvested, washed with 1X phosphate buffered saline (PBS) solution and then fixed with 2.5% glutaraldehyde. Following three rinses with distilled water, the samples were dehydrated through a series of graded ethanol solutions, and then air-dried overnight. Dry constructs were sputter coated with gold and observed under the FESEM at an accelerating voltage of 5.0 kV.

3.7 HDFs morphology, viability, attachment and count studies

Cell morphology was studied using the FESEM and Laser Scanning Confocal Microscopy (LSCM, Leica, Germany). HDFs were seeded at a density of 10,000 cells per well onto the PCL/gelatin scaffolds and TG-NF constructs. Such low densities were used for proliferation studies because there would be more available areas for cells to proliferate, and also to avoid reaching confluence prematurely. For LSCM observations, the seeded HDFs were stained with green fluorescence probe 5-chloromethyl fluoroscien diacetate (CMFDA) and observed under the LSCM (excitation 492nm, emission 517nm). Cell viability and attachments were determined by MTS assay. The number of attached cells was also determined by cell counting.

3.8 MTS assay

To study cell proliferation on the substrates, viable cells were determined by using the colorimetric MTS assay (CellTiter 96® AQ_{ueous} Assay, Madison, WI, USA). The mechanism behind this assay is that metabolically active cells will react with the tetrazolium salt in the MTS reagent to produce a soluble formazan dye that can be absorbed at 492nm.

The substrates were rinsed with PBS, followed by incubation with 20% MTS reagent in serum-free culture medium for 3 hours. Thereafter, aliquots were pipetted into a 96-well plate. The 96-well plate was then placed into a spectrophotometric plate reader (FLUOstar OPTIMA, BMG Lab technologies, Germany) and the absorbance at 492nm of

the content of each well was measured. These absorbance values are subsequently used to plot the growth of HDFs and gauge its concentration. However, they do not represent the number of HDFs present. Such absorbance values are also known as optical densities.

3.9 HDFs counting

In order to count the number of viable cells attached to the PCL/gelatin scaffolds and TG-NF constructs, the substrates were harvested, washed with PBS to remove non-adherent cells, and then incubated in 0.5 ml of 1x trypsin at 37^{o}C for 5 min. The trypsinization process was stopped by adding 0.5 ml of DMEM to each sample. The cell numbers were then counted using a hematocytometer and microscope.

3.10 Dual side HDF growth on PCL/gelatin scaffold

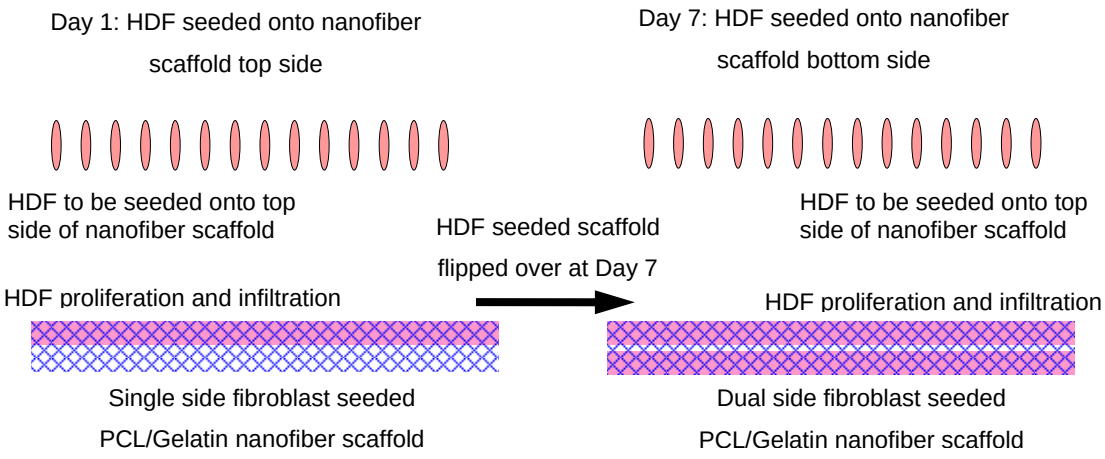

Figure 3.3: Schematic of proposed dual side HDF growth on a nanofiber scaffold

In this approach, conventional methods of cell seeding and culture works were performed on one side of the nanofiber construct and allowed for cell proliferation and penetration for up to 7 days. Subsequently, the construct was flipped over and the entire process was repeated for another span of 7 days. Cell viability assays were conducted through the entire 14-day span at regular time interval to ensure stability and consistency in cell proliferation. FESEM and LSCM micrographs were also taken to observe HDF population on each side of the scaffold.

Chapter 4: Results and Discussion

4.1 Morphology of electrospun PCL/gelatin nanofibrous scaffold

Morphological image of the PCL/gelatin nanofiber, which was similarly electrospun onto Tegaderm^{TM} wound dressing to form the TG-NF construct, were taken using the FESEM and shown in Figure 4.1. The micrograph image depicted randomly interconnected structures and seemingly smooth morphology of the electrospun PCL/gelatin nanofiber. The diameter of the nanofiber estimated to be in the range of 300 – 600 nm (90% nanofibers) with a mean diameter of 470 ± 120 nm by using the image analysis software (ImageJ, National Institutes of Health, USA). The PCL/gelatin nanofiber mat with a thickness of around 28 μm was obtained through 2.5 hours of electrospinning. Knowing the estimated bulk density of the PCL/gelatin (1.2–1.3 g/cm3), along with the calculated value of its apparent density, the porosity of the PCL/gelatin naofibrous scaffold could subsequently be calculated. These results are summarized in Table 1.

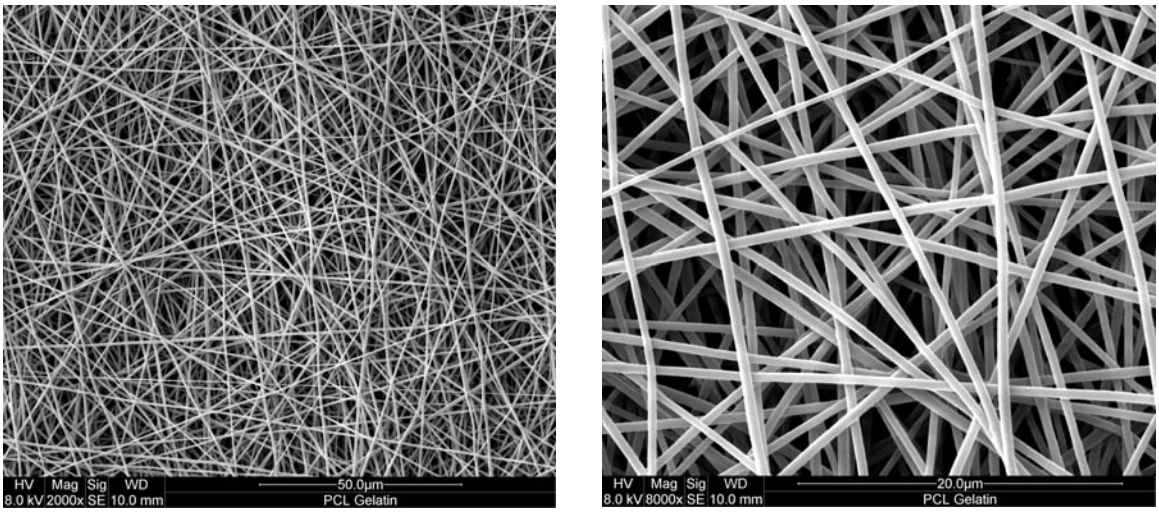

Figure 4.1: FESEM micrographs of PCL/gelatin nanofibrous scaffold

Porosity is viewed as a crucial parameter for tissue engineered scaffolds. Such nanofibrous scaffolds which would be used for skin reconstitution should largely be made porous. The porous nature of the scaffolds would then be beneficial for cellular infiltration and proliferation. On the other hand, the porous structure should continue aiding in hemostasis, as well as ensuring sufficient gaseous and nutrients exchange for wound healing.

According to He *et al* [10], the preferred porosity of scaffolds used for cellular penetration should generally be within the range of 60% to 90%. This could easily be achieved via phase separation and solid free-form fabrication techniques. The porosity of the electrospun PCL/gelatin nanofibrous scaffolds in this study is estimated to be around 60-70%, comparable to the techniques mentioned previously. The pores generated within the scaffold structure for cellular in-growth are formed by nanofibers lying upon each other which are different from isotropic pores made by using particles or bubbles when the scaffold is solidified. [10] Previous studies on PCL/gelatin nanofibers have also shown that the gelatin component of the co-polymer is gradually dissolved during cell culture, thus creating more spaces for cell migration. Similarly, the good elongation and deformation properties of gelatin provides easier opening of spaces for cell penetration to deeper level of the scaffold. [16]

It is not easy to create well defined pore sizes through the electrospinning technique because of the randomly deposited fibers. However, the overall network architecture

structure fabricated best mimic the natural ECM and this provides an advantage in its application as tissue engineered scaffolds. [10]

Table 1: Diameter, thickness, apparent density and porosity of PCL/gelatin nanofibrous scaffold

Diameter (nm)	Thickness (μm)	Mass per unit area (mg/cm^2)	Apparent density (g/cm^3)	Porosity (%)
470 ± 120	28 ± 6	1.21 ± 0.13	0.43 ± 0.06	62 – 75

Data are representative of three independent experiment and all data recorded as mean values ± SD (n=3)

4.2 Cell proliferation studies on TG-NF construct and PCL/gelatin scaffold

HDF proliferation on the TG-NF constructs was studied at Days 1, 3, 5, and 7, with results shown in Figures 4.2 and 4.3. The PCL/gelatin scaffolds were used as control. Both the optical density and number of cells were noted to have increased significantly through the 7-day span, demonstrating that cell proliferation occurred successfully on both substrates.

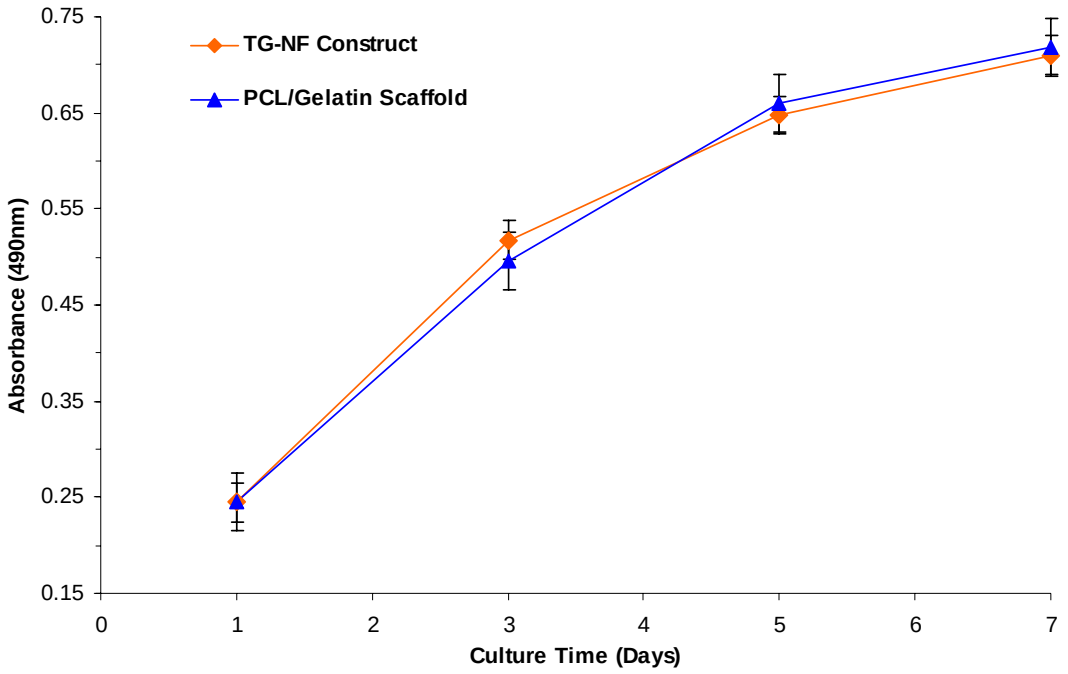

Figure 4.2: HDFs proliferation results (Cell viability)

Data are representative of three independent experiment and all data points plotted as mean values ± SD (n=3)

In Figure 4.2, it was observed that optical density of HDFs on both substrates kept increasing. The HDFs would continue to proliferate till confluence by which there would be no further available surface area on the constructs for further proliferation. This result is substantiated by the observations made in Figure 4.3, where cell counts were also noted to have increased substantially through the 7-day span.

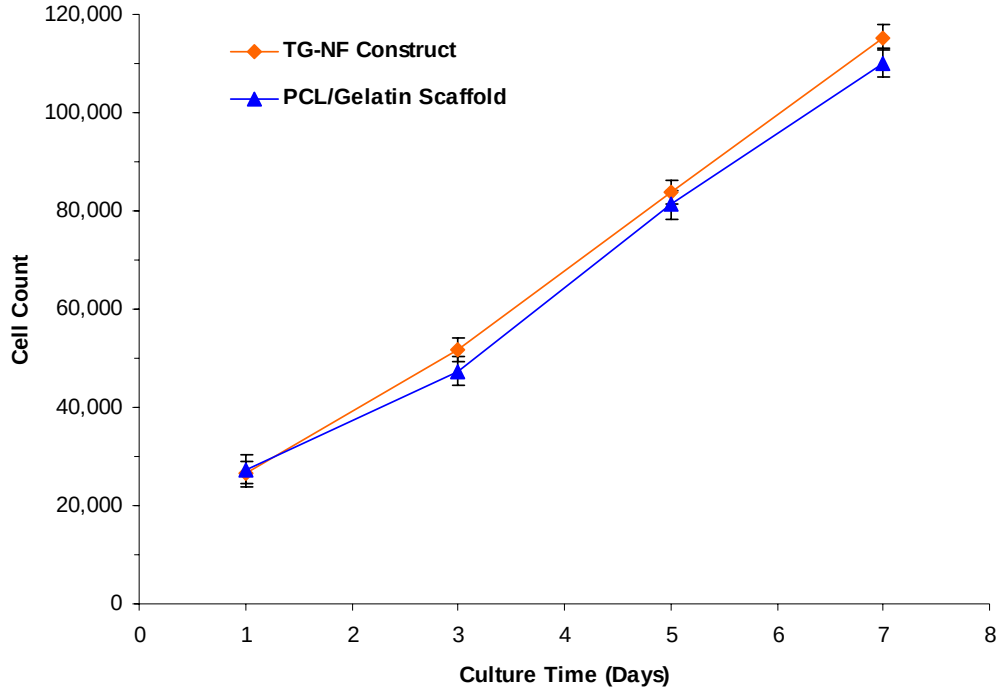

Figure 4.3: HDFs proliferation results (Cell counting)

Data are representative of three independent experiment and all data points plotted as mean values ± SD (n=3)

At every time point, the numbers of cells present on the PCL/gelatin scaffold and TG-NF construct were comparable, demonstrating that cell growth were comparable on both types of substrates. The presence of the Tegaderm^{TM} wound dressing in combination with

the nanofiber scaffold does not have any negative influence on cell proliferation. Instead, it does not hinder cell growth and is able to function in its role as a synthetic epidermis for protection of wound site. Therefore, it can be concluded that the TG-NF construct is a suitable non-toxic substrate for cell growth and proliferation.

4.3 Cellular morphology on TG-NF construct and PCL/gelatin scaffold

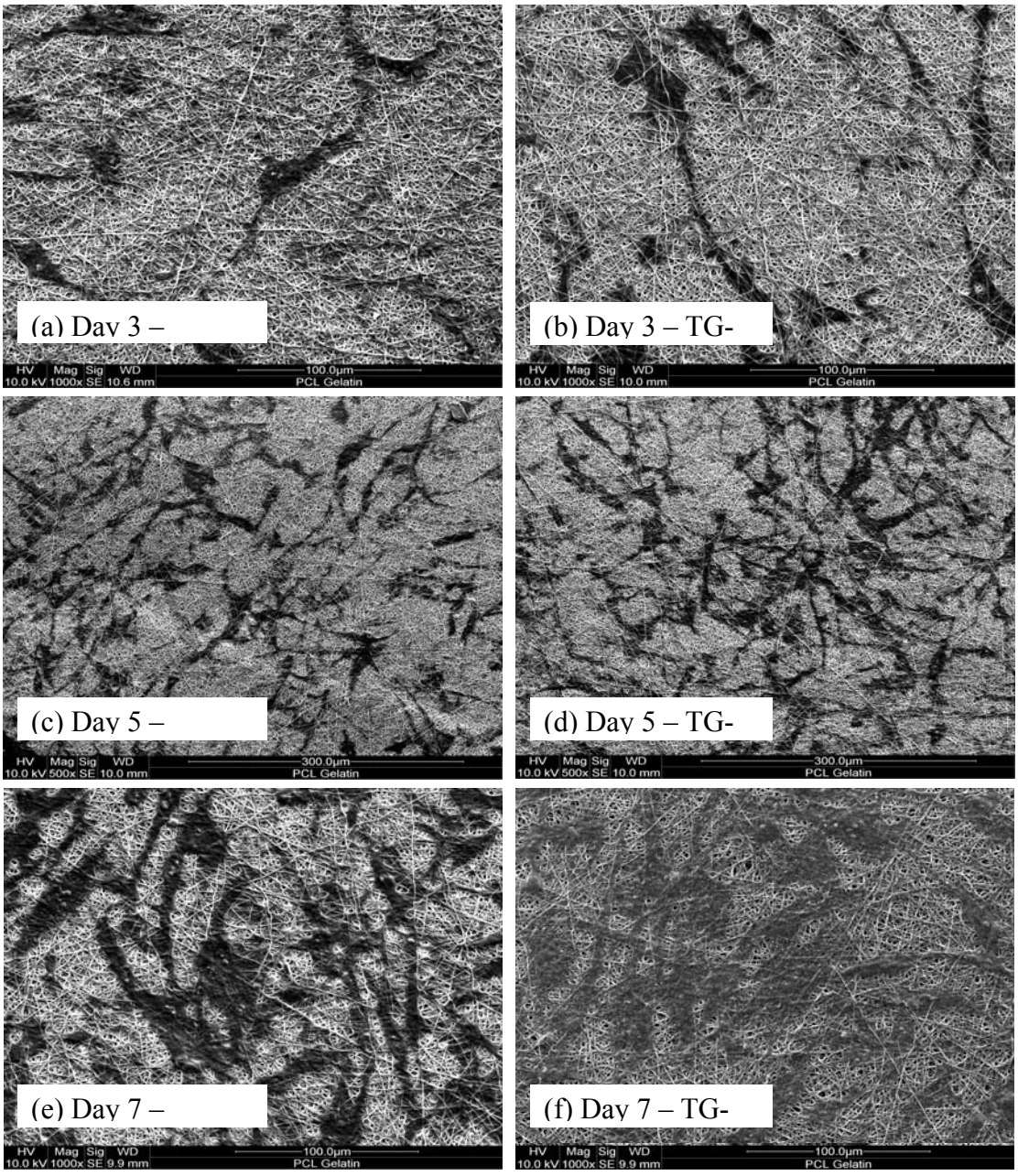

Figure 4.4: FESEM images of HDFs on PCL/gelatin scaffolds and TG-NF constructs: (a) Day 3 – PCL/gelatin scaffold (1000x). (b) Day 3 – TG-NF construct (1000x). (c) Day 5 – PCL/gelatin scaffold (500x). (d) Day 5 – TG-NF construct (500x). (e) Day 7 – PCL/gelatin scaffold (1000x). (f) Day 7 – TG-NF construct (1000x).

Cell morphology on the PCL/gelatin scaffolds and TG-constructs was studied by FESEM at Days 3, 5 and 7, with the results shown in Figure 4.4. Confluency of HDFs on the TG-NF constructs and PCL/gelatin nanofiber scaffolds were then estimated via visual observation and inspection. It was observed that at Day 3 (Figures 4.4a and 4.4b), HDFs only reached approximately 20% confluence. This was due to the low HDF seeding density used in this study in order to observe proliferation over a 7-day span. The cells were seen to be characteristically elongated and stretched across the nanofibrous substrates during the course of proliferation. Subsequently at Day 5 (Figures 4.4c and 4.4d), it was observed that the HDFs had increased in numbers and reached about 50% confluence on both substrates. HDF proliferation and growth continued progressively and by Day 7 (Figures 4.4e and 4.4f), the cells had already increased significantly in numbers, reaching a confluency of about 70%. Both the PCL/gelatin scaffold and TG-NF construct were almost completely proliferated with HDFs. From the FESEM micrographs taken, it was established that there was successful HDF proliferation and adherence on both substrates. Comparing the FESEM micrographs taken for both the PCL/gelatin scaffolds and TG-NF constructs at each time point, it was observed that the density of HDFs on both substrates was always comparable and that the visible confluence of the cells reached was almost equivalent. From this experimental data, it can be seen that the TG-NF construct can be considered a suitable host substrate for fibroblast population, with results of cell proliferation very much comparable to that of the PCL/gelatin nanofibrous scaffold.

With the aim of establishing the nanofiber construct as an autogenous fibroblast populated three-dimensional scaffold for wound healing purposes, there needs to be cell migration into the scaffold structure and not just on the substrate surface. Such maximized cellular in-growth would result in close biomimicry of the natural extracellular matrix (ECM). From the FESEM micrograph shown in Figure 4.5, it was observed that there was not much significant fibroblast infiltration within the scaffold structure. Noticeably, there was slight penetration within the top most layers of nanofibers, but not in-depth enough to be justified as a well populated three dimensional stereo-structure. The main reason being that the pores formed in the nanofiber scaffolds were still relatively small in comparison with the fibroblast cells, which are of sizes 10-100 micron. To overcome this problem, methods such as fiber leaching was proposed previously [37] to create micropores to encourage cell infiltration. However, in our study here, we propose a new approach to resolve this problem. Fibroblasts are cultured on both sides of a relatively thin construct of nanofibrous scaffold, such that the cells can proliferate, populate and penetrate from both sides (Figure 3.3). This would increase the cell loading capacity as compared to that of the usual loading cells on only one-side of the scaffold.

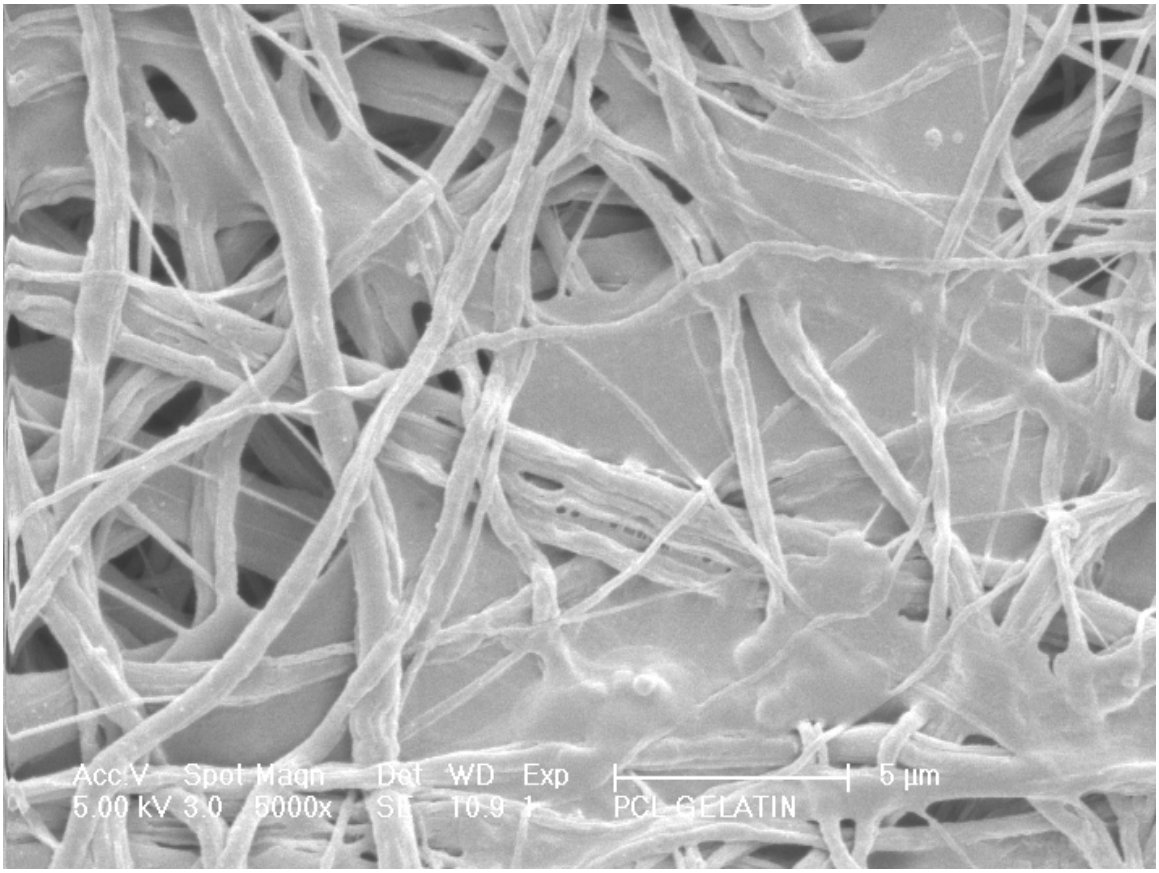

Figure 4.5: FESEM image showing slight penetration of HDF within top most layers of nanofibers (Magnification 5000x)

4.4 Dual side HDF growth on PCL/gelatin scaffold

In this experiment on dual side HDF growth on PCL/gelatin scaffold, FESEM and LSCM micrographs were taken to observe HDF population on each side of the scaffold. HDFs were seeded onto one side of the scaffold and allowed for cell proliferation for up to 7 days. This side of the scaffold is termed "Top side". Subsequently, the scaffold was flipped over and HDFs were seeded again. This opposite side of the scaffold is termed "Bottom side". Thus, by the end of the 14-days span, the top side of the scaffold would have undergone 14 days of cell culture, compared with the bottom side which had

undergone 7 days. The viability assay results, FESEM and LSCM micrographs are shown in Figures 4.6 and 4.7 respectively.

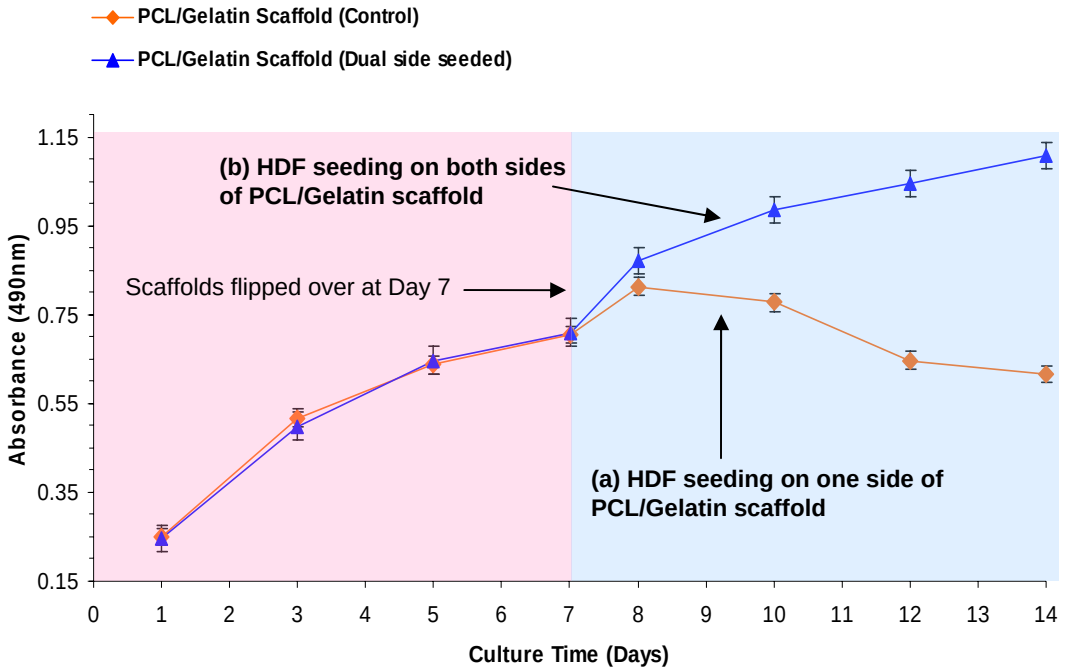

Figure 4.6: HDF proliferation results (Cell viability)

– (a) One side of nanofiber scaffold, and (b) Both side of nanofiber scaffold.

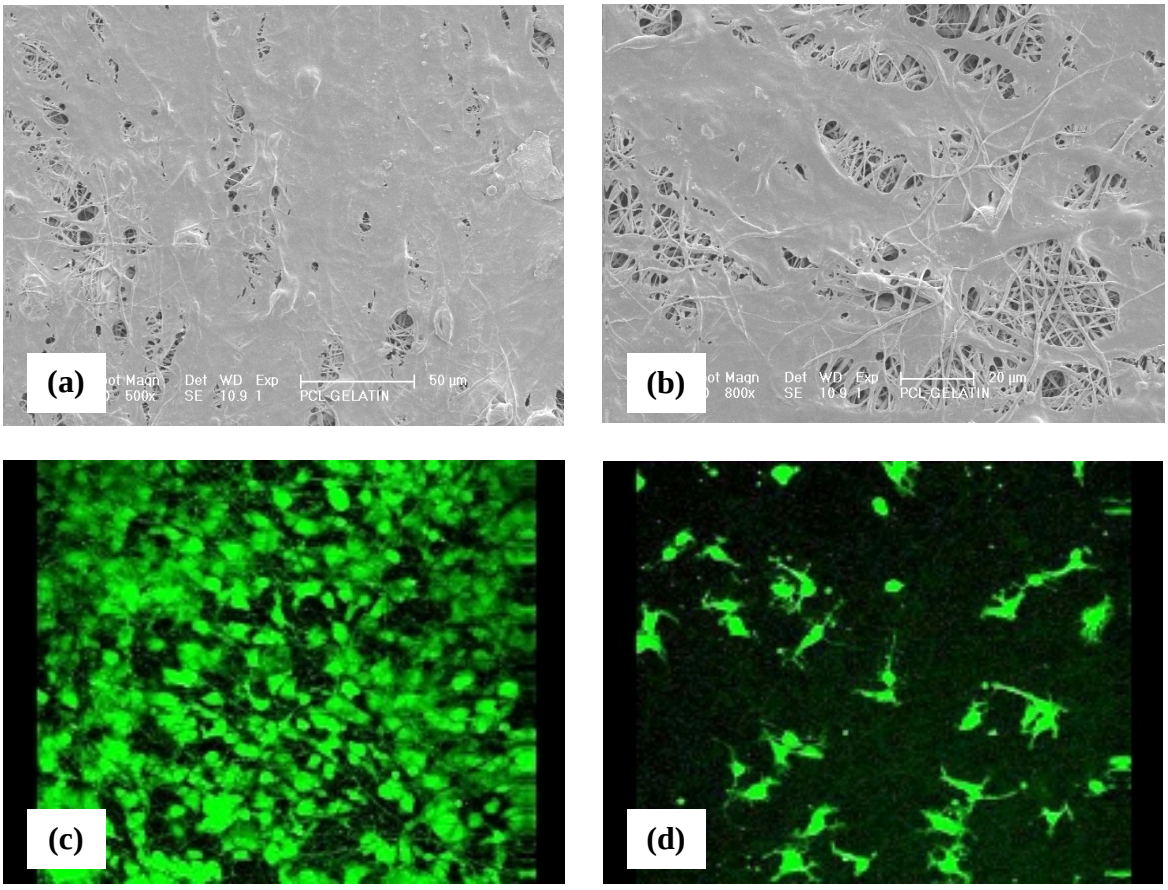

Figure 4.7: FESEM and LSCM images of HDF population on PCL/gelatin scaffold – Left: Top side; Right: Bottom side.

Results from the viability studies (Figure 4.6) show that the fibroblasts grow and proliferate well on both sides of the nanofiber scaffold. Optical density of the cells increased through the first 7 days span (Figure 4.6a). This result was similar to the ones obtained in the first part of the studies on TG-NF where HDF seeded PCL/gelatin scaffold was used as a control. Subsequently, the viability studies were conducted again on the opposite side. This time, the optical density was appreciably higher at the regular time points, as compared with the control PCL/gelatin scaffold with HDF seeded on one side only (Figure 4.6b). This happens because cell proliferation had now continuously

occurred on both sides of the scaffold and the flipping over of scaffold did not affect cellular activities. These results were justified from micrographs taken using the FESEM and LSCM as shown in Figure 4.7. Significantly at Day 14, it was observed from the FESEM micrograph that one side of the scaffold was very confluent with HDF population (Top side) while the other side (Bottom side) was distinctly 70% confluent. In this case, the former is the side which was seeded with HDF earlier and the latter being the side which had HDF seeded at a later phase. These separate images also showed that the fibroblast cells proliferated as usual on both sides of the scaffold without any compromise. Given that such a phenomenon happens on both sides of the nanofibrous scaffold and coupled with the thinness of the construct, a split-thinness fibroblast populated scaffold will now be possibly feasible.

Chapter 5: Conclusions

In this study, the objective of investigating the feasibility of the TG-NF construct as an effective TE scaffold for fibroblast integration and proliferation to establish a fibroblast populated dermal analogue was achieved. Results from experiments confirmed that HDF cells can grow and proliferate well on the TG-NF construct; in fact as well as on a usual nanofiber scaffold, which has been gaining recognition in recent researches as being a suitable biodegradable scaffold for skin substitutes. There is thus the added incentive of electrospinning nanofibers onto Tegaderm^{TM} wound dressing, which acts as a synthetic epidermis to protect both the wound and the fibroblast-populated nanofiber lattice from contamination and does not compromise HDF proliferation.

In line with developing a three dimensional scaffold construct for HDF infiltration, the fibroblast cells were successfully cultivated on both sides of a thin PCL/gelatin nanofiber scaffold. This allowed cell growth and penetration from both sides of the scaffold, thus maximizing cell loading and growth into the scaffold structure which mimics the natural ECM.

The data obtained from these experiments are an important first step in the development of nanofibrous scaffold as a suitable dermal analogue to assist in skin cover and regeneration. Ultimately, the nanofibrous scaffold is a thin one, and may be insufficient to replace the lost dermis in terms of thickness. Clinical studies downstream will investigate the feasibility of adding successive layers of the dual-sided fibroblast seeded scaffolds to reconstitute full dermal thickness (Autologous Layered Dermal

Reconstitution: ALDR) [46] before final application of keratinocytes, either as a thin split thickness skin graft or as unpolarized keratinocytes sprayed on in combination with fibrin glue, to reconstitute the epidermis and achieve full skin reconstitution.

Chapter 6: Recommendations

Through the course of this study, certain limitations and difficulties were encountered. Various means of improvement were also identified for future studies into this area:

1. Aligned or core-shell nanofibers – Using these approaches would result in better control over the porosity, pore sizes and structural properties of the scaffold. The resultant topography would better promote HDF adhesion, proliferation and integration [18].
2. Use of composite nanofibers – Nanofibers could be coated with suitable growth factors and proteins to promote cell adhesion, proliferation and growth on and within the scaffold.
3. Autologous Layered Dermal Reconstitution (ALDR) – Further clinical investigations will be made into the layered application of the TG-NF construct to build up the injured dermis prior to re-epithelialisation using split-thickness skin graft.
4. Composite cultured skin – By applying the concept of dual side cell seeding on nanofibrous scaffold, a composite skin structure made up of layered keratinocytes-scaffold-fibroblast could be developed.
5. Variance in cell types – Since HDF has already been used in this study for dermal regeneration, a similar approach could be made applicable using other cell types e.g. osteoblasts and SMCs. If proven successful, this approach could be expanded towards applications in TE bone or muscle regeneration.

6.1 Autologous Layered Dermal Reconstitution (ALDR)

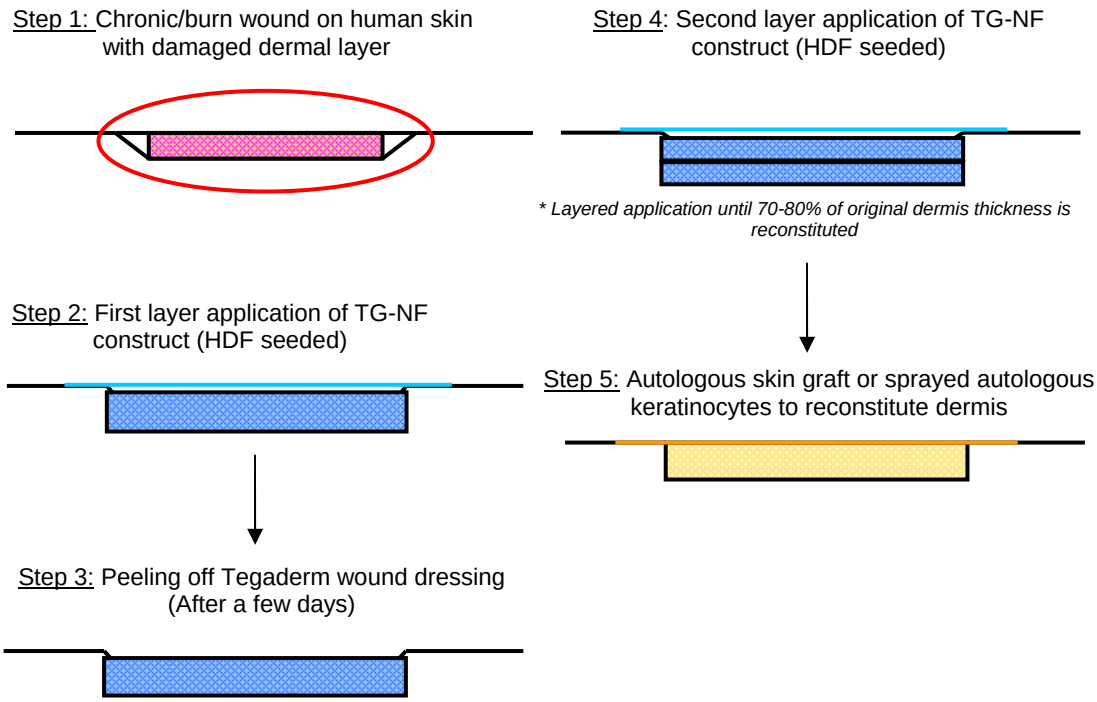

Figure 6.1: Schematic of Autologous Layered Dermal Reconstitution (ALDR)

In the process of wound healing and dermal reconstitution, it is important that the replacement analogue has a good "take" onto the wound site so as to facilitate and enhance re-epithelization. However, current artificial dermal analogues have significant thickness such that the "take" rate is relatively lower. In line with the thinness of the TG-NF constructs and three-dimensional fibroblast incorporated scaffold, a new technique of approaching dermal wound healing (ALDR) is proposed so as to achieve better "take" onto wound sites and also obtain better wound healing results [46]. In the proposed schematic illustration shown in Figure 6.1, layered applications of the constructs are

continued, with subsequent removal of the Tegaderm^{TM} wound dressing, until at least 70-80% of the original dermal layer has been reconstituted. A split thinness autograft or sprayed kertinocytes can then be applied to reconstitute the remaining epidermal layer. The ALDR approach to dermal wound healing is novel and unique and further clinical testing is required to provide actual results [46] so as to explore its feasibility.

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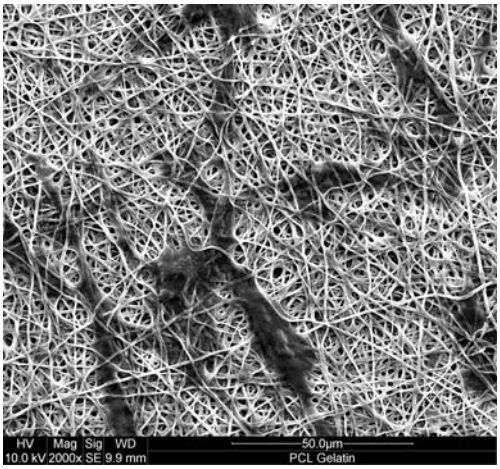
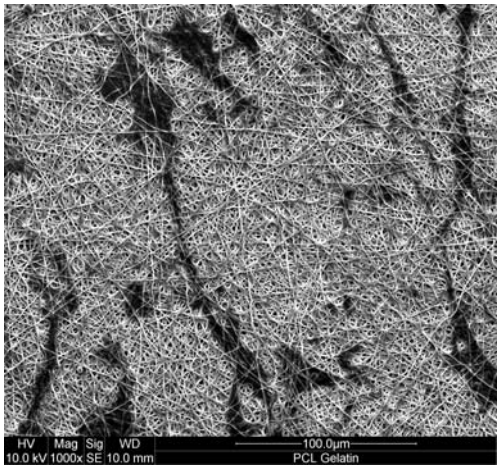
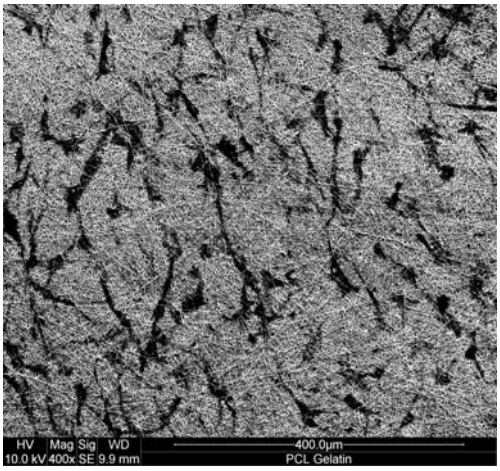
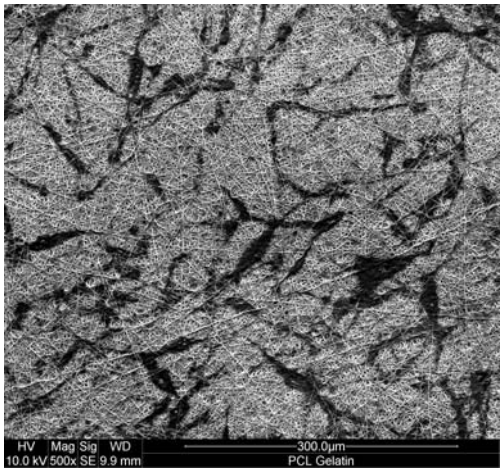
Appendix A: Morphological images of cell growth



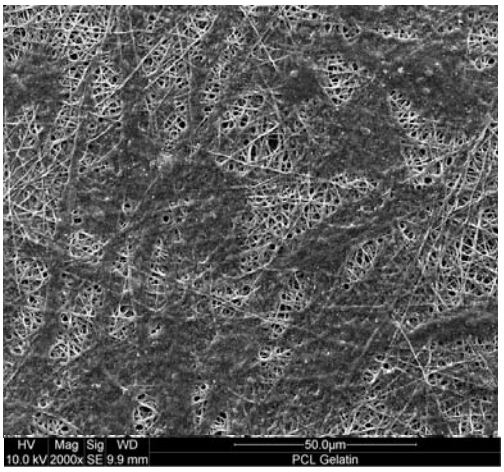


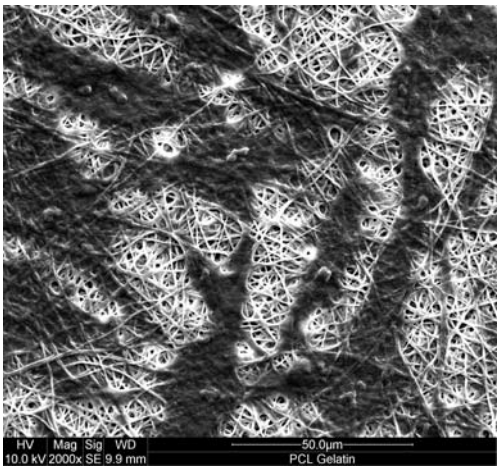
Day 3: PCL scaffold (left) and TG-NF construct (right)





Day 5: PCL scaffold (left) and TG-NF construct (right)





Day 7: PCL scaffold (left) and TG-NF construct (right)