Development of a biodegradable membrane to be used with skin explants for full thickness skin defect reconstruction

Thesis submitted to the University of Sheffield for the degree of Doctorate of Medicine

Department of Oncology and Metabolism
And
Department of Materials Science and Engineering

Miss Kavita Sharma
MBChB, MRCS

December 2018

Acknowledgements

This research would not be possible without the guidance and support of Professor Sheila MacNeil. From my introduction to the lab over seven years ago, to the hours of feedback, critical appraisal of my writing and experiment planning that made this work possible, I am very grateful. Your vision for the future of tissue engineered skin is pioneering. From you I have learnt the science and intended clinical applications of biomaterials, which will undoubtedly positively influence my patient care in the future.

I would like to thank the Sheffield Teaching Hospitals for their support which made this degree a reality especially Mr David Ralston, Consultant Burns Surgeon and Mrs Victoria Giblin, Consultant Plastic Surgeon.

I would also like to acknowledge my colleagues in the MacNeil research group: Dr Naside Mangir, Dr Sabi Roman and Dr Athasham Raza, who went out of their way to help me acquire the necessary skills to adopt a questioning and logical approach to my work, perform my experiments and make sense of my results. I would also like to thank Dr Anthony Bullock, for teaching me electrospinning and cell culture and reading through my result chapters.

This work, forms one aspect of my career as a developing Plastic Surgeon. It reflects my determination to contribute to a field of research that I developed a keen interest in as a junior doctor, to understand tissue engineering, a technique which will become reality in generations to come.

Table of Contents

Awards and Presentations

Awards

Maurice Wohl Research Fund from the Royal College of Surgeons Edinburgh, UK,

Presentations

The optimisation of a one stage tissue engineered skin substitute using fibrin for frill thickness skin defect reconstruction. British Burn Association Meeting 2018 (Oral): 11th-13th April 2018.

Abbreviations

%	percentage
α	alpha
β	beta
λex	excitation wavelength
λem	emission wavelength
µg	microgram
µl	microlitre
µM	micromolar
µm	micrometer
µs	Microseconds
2D	two dimensional
3D	three dimensional
316L	Steel containing less carbon
Ab	antibody
AA	l-ascorbic acid
A2P	ascorbate-2-phopshate
BMI	body max index
BSA	bovine serum albumin
CEA	Cultured epithelial autografts

cm	centimetre
cm^2	square centimetre
CO2	carbon dioxide
Col	collagen
ºC	degree celsius
CMFDA	CellTracker green
CMTPX	CellTracker red
D	dextro
DAPI	4',6-diamidino-2-phenylindole
DCM	dichloromethane
DED	De-epithelised dermis
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulphoxide
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor (Human recombinant)
F-12 HAM	Cell culture media
FBS	fetal bovine serum
FDA	Food and Drug Administration

FITC	fluorescein isothiocyanate
FGF	fibroblast growth factor
g	gram
pg	Pico gram
G	Gauge
GMC	General Medical Council
GMP	good manufacturing practice
h	hour
H&E	haematoxylin and eosin
HCL	Hydrochloric acid
Hz	hertz
HTA	Human Tissue Authority
I.U	International units
I3T3	Irradiated mouse fibroblast feeder layer
IMS	industrial methylated spirit
kV	kilovolts
L	levo
M	molar
mg	milligram
min	minute

mL	millilitre
mM	millimolar
mm	millimetre
MMP	matrix metalloproteinases
No.	number
nm	nanometre
nM	nanomolar
N	newton
N/mm	newton/millimetre
N/mm^2	newton/square millimetre
NaCl	Sodium Chloride
NaOH	Sodium hydroxide
NHS	National Health Service
OCT	optimal cutting temperature compound
OH	Alcohol (methanol)
P	passage
PBS	phosphate-buffered saline
PGA	poly-glycolic acid
PHBV	Polyhydroxybutyrate/Polyhydroxyvalerate (PHBV) 12% biopolymer

pH	potential of hydrogen
PLA	poly-(L)-lactic acid
PGLA 75:25	poly- (D, L-lactide-co-glycolide) lactide: glycolide
rpm	revolutions per minute
sec	second
SEM	scanning electron microscopy
SLM 125	Selective Laser Melting Machine
T75	Flask used for cell culture
TGF-β1	transforming growth factor beta-1
TNFα	tumor necrosis factor α
TT	3,3,5 –Tri-iodo-L-thyronine (T_3)/Apo-Transferrin
UTS	ultimate tensile strength
V	volts
W	Watts
w/v	weight/volume
YM	Young's modulus

List of Figures

List of Tables

Abstract

Tissue engineered skin has advanced significantly in creating a substitute to mimic human skin. Advances in biomaterial sciences and a better understanding of wound healing have been the catalyst for development of tissue-engineered skin substitutes. We aimed to develop and evaluate a biodegradable dermal alternative composed of FDA approved polymers consisting of polyhydroxy-butyrate-co-hydroxyvalerate (PHBV) (nanofibres) and polylactide-co- glycolide (75:25) (microfibers)to be combined with skin explants as a one-step approach for full thickness skin defects reconstruction.

The scaffolds consisted of an upper and under layer of PGLA 75:25 which is known to facilitate the migration of epithelial cells and a middle layer of PHBV to act as a basement membrane separating the epidermis from the underlying dermis. The undulating topography of the dermo epidermal junction was replicated by templating these undulations on the middle layer of scaffolds using metal collectors. The rete ridges are thought to create a microenvironment for stem cells to reside. To assess this, we undertook Ki67 immunohistochemistry which demonstrated that there was some activity in the trough of the undulations but this was not convincing.

As part of the one step approach we used skin explants to populate the scaffolds as opposed to isolated epithelial cells which requires planning and appropriate staff and facilities. We investigated the ability for cells from skin explants to migrate from the cut edges of onto the electrospun scaffolds compared to using isolated keratinocytes and fibroblasts. It was found that cells from the explants will migrate along the fibres of the scaffold but not as much as using isolated cells in the same time period in terms of metabolic activity, collagen production and qualitative assessments using DAPI and H&E.

The ability for optimization of migration of cells from the skin explants was assessed using fibrin, a biological tissue glue in current clinical use in its ability to retain the explants on the scaffold. We found that a fibrin constituent combination of thrombin 2.5 I.U and fibrinogen 18.75mg allowed manipulation of skin explants on the scaffold and migration of cells along the fibres. We also found that explants with less dermis had more outward cellular migration from their cut edges compared to those with more dermis. Finally, we assessed the ability for the constructs to have another layer of scaffold on top to aid cellular migration but found that this did not significantly influence outgrowth.

Furthermore, we tested the effect of keratinocyte migration from skin explants in the presence of isolated fibroblasts pre-cultured on scaffolds as an in vitro wound bed model. This revealed significantly increased metabolic and proliferative activity of constructs in production of collagen. Although in our model for clinical translation we do not intend to use isolated fibroblasts to pre culture scaffolds, it was felt that this was the closest to a wound bed model as the scaffolds would be placed onto a prepared wound bed. Immunohistochemistry was used to demonstrate that keratinocytes were migrating from the cut edges of the skin explants and confocal imaging demonstrated that the cells penetrated the layers of the trilayer scaffold.

In summary were able to design a suitable synthetic dermal scaffold and populate it with cells from skin explants. To facilitate this, we propose the use of fibrin and skin explants with less dermis to facilitate skin cell migration. Animal studies are now needed to further define parameters of the use of this in the clinical environment.

Chapter 1

INTRODUCTION

1.1 Structure of Human Skin

The skin is the largest organ in the body, covering around $2m^2$ in an average adult(1). It functions as a barrier protector against external microorganisms, water loss and has a role in thermoregulation. Skin loss, when significant enough can activate the systemic inflammatory response resulting in shock and multi organ dysfunction as a result prompt wound coverage is a key objective in many aspects of plastic, burns and reconstructive surgery. In order to augment wound healing by creating a skin substitute we must firstly understand the microstructure and topographical anatomy of skin, which allows it to fulfil its most pertinent role as an anatomical barrier against evaporative and heat losses and the entry of micro-organisms.

Human skin can be thought of as having three identifiable layers. The epidermis or the waterproofing layer; the dermis or the supportive layer and the hypodermis or the padding layer (Figure 1.1). Although these layers have been listed distinctly, they do not function independently and are interlinked by a series of complex protein systems that determines the skin's functionality as an organ.

Labels in figure:
- Epidermis
- Dermis
- Subcutis
- Keratin layer
- Cells in the epidermis
- Sebaceous gland
- Dermal duct or eccrine sweat gland
- Arrector pili muscle
- Hair follicle
- Nerve fiber
- Deep blood vessels
- Collagen and elastin fibres
- Adipose tissue
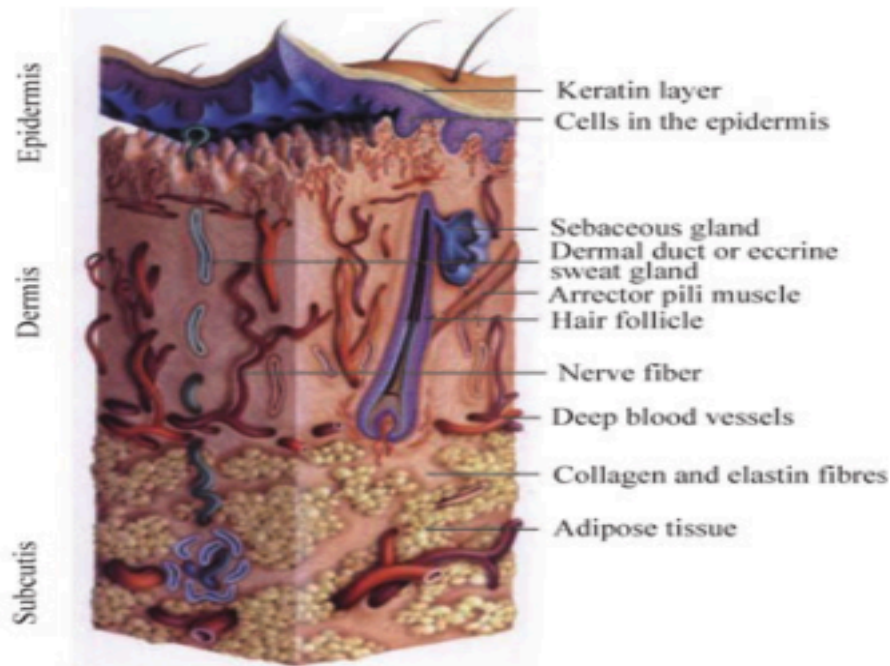
Figure 1.1 *Schematic Representation of layers of human skin. Source: Bhusan et al 2010(2). Reproduced with permission from John Wiley and Sons.*

1.1.1 Epidermis

The epidermis consists mainly of stratified keratinocytes. The layers include the strata basale, spinosum, granulosum and corneum. The epidermis continuously regenerates itself from the basal layer over approximately four weeks to form anuclear keratin cells that make up the stratum corneum. Stratification is an essential feature of the topography of the epidermis, as cells exist in various stages of proliferation and differentiation in the differing layers. It is this combination of differentiation and stratification that is responsible for the barrier function of the skin(3). Therefore, any skin replacement should facilitate

the migration, proliferation and stratification of keratinocytes in order to sustain a functional epidermis.

1.1.2 Basement Membrane

The basement membrane, located between the epidermis and the dermis is a thin layer of specialized extracellular matrix onto which the epidermis is attached. It serves to tightly link the epidermis to the dermis; provides a barrier against epidermal migration; determines the polarity of the epidermis and acts as the middleman communicator between the epidermis and dermis to maintain haemostasis, which is one of the main functions of the skin(4).

Inoue demonstrated that the basement membrane could be morphologically distinguished into four layers: the plasma membrane of the basal keratinocytes; lamina lucida; lamina densa and sublamina densa. These all act collectively to augment the keratinocyte –dermis attachment(5). The lamina lucida is the interface between the basement membrane and the basal keratinocytes. In this layer, Integrins link hemidesmosomes, which are stud like structures attached to the plasma membrane of the basal keratinocytes to the extracellular matrix of the basement membrane. These integrins are known as laminin 332 and 511(6). The lamina densa provides mechanical strength and the middle layer

that forms a sheet like structure comprising of type IV collagen, nidogens and perlecan(7). The sublamina densa (lamina fibroreticularis) is the deepest layer of the basement membrane which contains collagen VII that extends through fibrils and insert into plaques in the reticular dermis(8).

1.1.3 Rete ridges

The anatomical structure of skin reflects the mechanical stress that cells in that region are likely to experience. The basement membrane is topographically organized into peaks and troughs in the post-natal skin. These develop mid-gestation where prior to this; the dermal epidermal junction is flat(9).These structures referred to as rete ridges range from 50-400 micrometres in width and 50-200 micrometres in depth(10)'(11) (Figure 1.2).

Epidermal stem cells lie in specific locations relative to the rete ridges depending on their location in the body. In the palmoplantar regions the peak of the rete ridges are those areas where the epithelium projects most deeply into the dermis. Lavker and Sun observed that there were different shapes and rates of cell division in the basal keratinocytes based on whether they were located in the tip or trough of the rete ridges. They concluded that the stem cells might inhabit the tips only of the deep rete ridges(12). However in non- palmoplantar

skin the stem cells seem to inhabit the troughs of the rete ridges i.e. where the dermis comes closest to the skin(13).

The rete ridges are an important feature in the topography of the dermal-epidermal junction. They provide structural integrity for the epidermal-dermal interface and define the cellular microenvironments, which then determines the functioning of the basal and suprabasal keratinocytes in these regions. Studies have demonstrated, using biopsies followed by histological analysis, that this specialized 3D topography is not present in the dermal-epidermal junction of tissue-engineered bio-substitutes, which maybe a limiting factor in the ultimate long term aim of any tissue replacement- to create a structure that ultimately will look and function like its intended replacement (14).

Interfollicular epidermis

Bulge area adjacent to the pilosebaceous unit

Trough of rete ridge
Peak of rete ridge
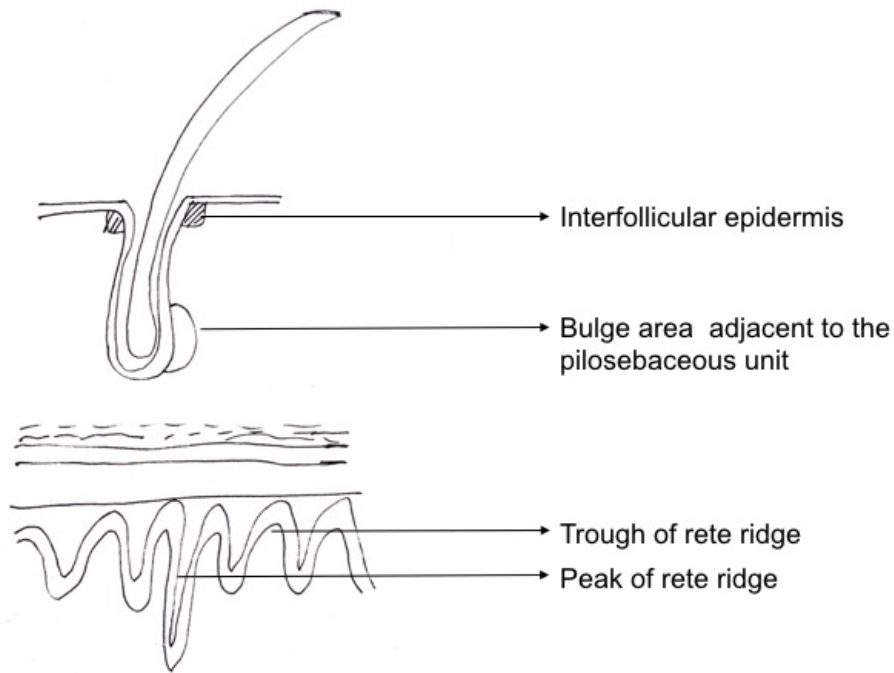
Figure 1.2a *Sketch of Rete Ridges and Stem Cell locations in the Dermis*

Undulating pattern of the Epidermal - Dermal Interface
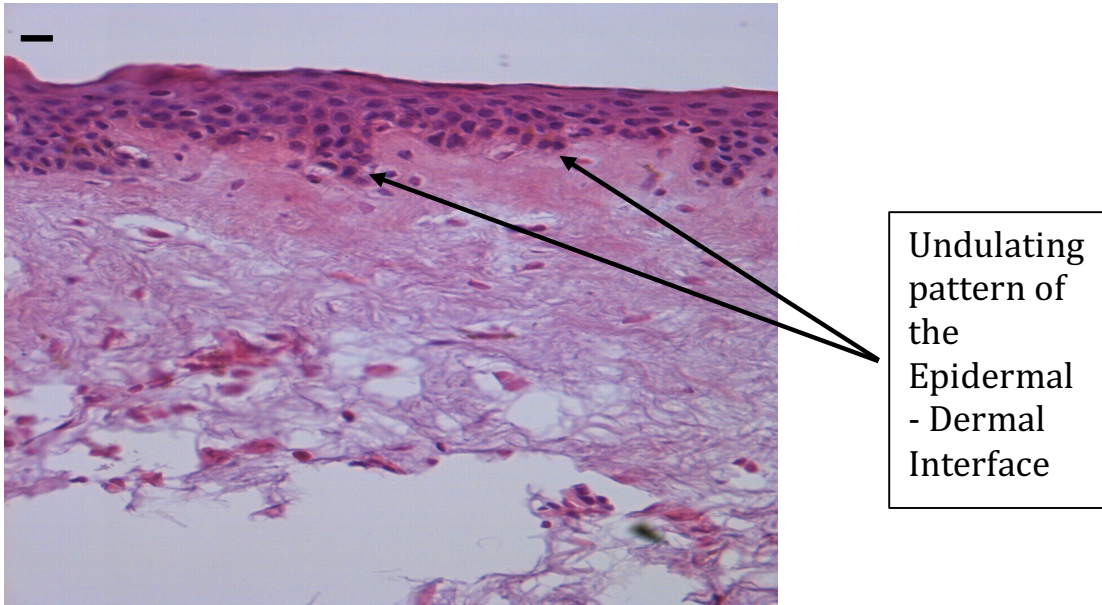
Figure 1.2b Histology image (Haematoxylin and Eosin staining) of a section of abdominal skin. This illustrates the various layers; epidermis (E), dermis (D) and the rete ridges (RR). Scale bar represents 100 μm.

1.1.4 Dermis

The dermis is divided into the papillary (uppermost) and reticular (lower) layers.

The papillary layer is undulating and intertwines with the rete ridges of the epithelium at the dermal-epidermal junction. Molecularly it consists of loosely arranged type III collagen fibres.

The reticular dermis is composed of a dense irregular meshwork of tightly packed type I collagen fibres elastin and glycosaminoglycans (extracellular matrix). Fibroblasts in the dermis are primarily responsible for the synthesis of the extracellular matrix. The reticular dermis provides skin with elasticity or resilience to deformity. Therefore, any skin substitute must have a robust

dermal component, which can support and facilitate collagen deposition and possess the tensile strength to allow the skin to maintain its mechanical and elastic properties.

1.1.5 Hypodermis

The hypodermis lies just under the dermis separated from it by a thin layer of superficial fascia. It contains variable amounts of adipose tissue depending on the part of the body that is being examined. This subcutaneous fat layer gives a smooth contour to the body. When it is lost for example in traumatic injuries, lack of regeneration results in contour defects to that particular area.

1.1.6 Blood Supply to the Skin

There are two horizontal plexuses or vascular trees that supply the skin. The upper one lies at the level of the papillary dermis and the lower lies at the dermal-hypodermal junction. Communication occurs between these 2 plexuses by way of paired arterial and venous networks. It is therefore essential that a skin substitute should be able to support and encourage timely vascularization which is generally accepted as 5 days clinically in order to ensure take (defined

as integration of the material to the underlying wound bed) and ultimately a successful skin replacement.

1.2 Characteristics of cells used to populate the electrospun scaffolds

1.2.1 Keratinocyte biology

In order to produce a scaffold that supports the growth of keratinocytes and fibroblasts it is essential to understand their behaviour, organization and requirements. Keratinocyte migration occurs in wound healing after about 18-24 hours. Migrating keratinocytes are morphologically altered in order to aid this. Some of the changes include disassembly of the links between the basement membrane and the keratinocytes and retraction of intracellular filaments to allow propagation and formation of peripheral cytoplasmic actin filaments (lamellopodia). Epithelization is controlled by communication between three main sets of molecules: growth factors, integrins and matrix metalloproteinases (MMPs)(15). These systems all integrate to form a migrating sheet of keratinocytes at the edge of the wound. The leading edge of this advancing sheet shows no mitotic activity. Instead the suprabasal keratinocytes are the first to migrate onto the wound; this reduces the number of cells and act as a stimulus for cell proliferation. Migrating keratinocytes produce MMPs, which loosens the adhesion of the keratinocyte to the dermal matrix thereby

allowing migration and re-epithelization. Once re-epithelization is complete the keratinocytes revert to their normal phenotypes, which is initiated by the process of contact inhibition(16).

1.2.2 Fibroblast Biology

Fibroblasts are the principal cellular components of connective tissues and populate the dermis of the skin. They are elongated, spindle shaped cells that have a high proliferative and migration potential. They function not only to synthesize and secrete extracellular matrix molecules but also growth factors, cytokines and chemokines that contribute to immune regulation and wound healing by influencing the migration and proliferation of keratinocytes(17).

Historical data has demonstrated that fibroblasts cultured in a blood clot varied in shape from stellate to bipolar depending on the orientation of the fibrous network of the clot. This led to the conclusion that there was a dynamic interaction between cells and their physical environment(18). When fibroblasts are cultured within an extracellular matrix eg fibrin as is planned in this work they experience a richer, more complex physical environment and different geometry than cells on 2D surfaces. Cells do not only migrate but work to remodel the matrix. The mechanical signals from the remodelled matrix then feedback to influence the phenotype of the cell(19).

The role of keratinocytes and Fibroblasts in wound healing

Wound healing can be divided into different phases based on the time from injury. Initially there is haemostasis where a clot is formed. This clot is composed of both platelets and fibrin. The fibrin serves also as a meshwork to direct the movement of fibroblasts into the wound. The inflammatory phase is next where the main cell, the macrophage releases chemoattractant like basic fibroblast growth factor, IL6, TNF alpha to attract other inflammatory cells to the wound but also to encourage the migration of fibroblasts into the wound[20]. Following this the proliferative phase sets in, where fibroblasts produce not only collagen but extracellular matrix proteins and secrete factors to attract keratinocytes into the wound. This then becomes a feedback loop where migrating keratinocytes stimulate the increased activity of fibroblasts and vice versa. This continues until the remodelling phase which starts from week 6 and can last until 18 months. Here type III collagen is remodelled to type 1 with no net increase in the quantity of collagen in the wound. The keratinocytes would have established a waterproof epidermal layer. It is important to note that these phases and cells accompanying are not mutually exclusive but occur in a continuum.

1.2.3 Epidermal Stem Cell Biology

The skin continuously renews itself throughout life as part of its role as a barrier and in response to pathological states like injury and degeneration. It is able to undertake this due to the proliferating cells that reside there. There are two main types of proliferating cells: the stem cells (SC) and the transit amplifying cells (TA cells). TA cells are undifferentiated cells that can be thought of as a transition between SCs and differentiated cells. They function as a feedback loop to SCs, to drive continued regeneration and they are also believed to influence other cell types to create an optimal environment for regenerating tissue (21). Stem cells on the other hand are undifferentiated cells with a capacity for self-renewal and differentiation into multiple lineages(22). They receive their regulatory cues from neighbouring cells within a defined and tightly controlled local microenvironment called the stem cell niche (SC niche). Some of the cells that constitute the niche are differentiated daughter cells of the stem cells and provide feedback regulation to their parent SCs.

Stem cells within adult skin are known to be concentrated in a few distinct areas. The first area is the bulge area around the pilosebaceous unit and the second is the interfollicullar epidermis (areas of the epidermis that is located between the exit of the hair follicle from the dermis through the epidermis onto the surface of the skin)(23). The stem cells that reside in these two areas have distinct functions and reside in different microenvironments or niches that determine

and regulate much of their activity. The interfollicular stem cells contain progenitor cells that ensures cell renewal in the absence of injury. This is a key feature of the self-renewing capacity of the epidermis. Studies on the progenitor cells in the interfollicular epidermis demonstrate that the epidermis undulates to form deep epidermal ridges called rete ridges that extend into the dermis. One function of the rete ridges is to anchor the epidermis to the dermis, however it also serves to create a microenvironment for the IFE stem cells at the base of the ridges. This is a more protected site than other areas of the IFE(12). The stem cells that reside in the rete ridges have been investigated using cultured human interfollicular keratinocytes which have determined that cells expressing the highest level of ß integrins have the highest proliferative potential in vitro(24). These so called ß1 bright cells are found especially in the basal layers of the epidermis in clusters(25) and at the base of the deepest epidermal ridges of palmoplantar skin

The bulge area around the pilosebaceous unit contains multipotent stem cells that are activated primarily upon cutaneous wounding. They provide cells for hair follicle regeneration and repair of the epidermis. There are various methods and markers published to isolate and potentially label epidermal stem cells. In vivo stem cells express higher levels of integrins than TA cells(25). Conjugated antibodies against integrin and anti-CD71 have been used to indicate the

location of stem cells in the epidermis by some groups(26). Other markers found to be increased in the stem cell population of the epidermis include S100A4 and S100A6 proteins(27), K19(28), K15(29) and CD34(30). Although most of these antibodies are not useful in proving isolated stem cells by flow cytometry the CD34 antibody seems to be an exception(23).

1.2.4 The concept of Skin Explants being used to regenerate an epidermis

The work in this thesis investigates the use of skin explants as a source of fibroblasts and keratinocytes as opposed to using isolated laboratory cultured cells to populate the electrospun scaffolds. Skin explants are small squares (5x5mm^2) of skin containing both epidermis and dermis, devoid of subcutaneous fat. We aim to encourage fibroblast and keratinocyte proliferation by placing explants onto electrospun scaffolds.

This method of expanding fibroblasts for culture in the laboratory is supported from the literature as it is a commonly used technique for establishing fibroblast culture(31). Traditional methods of fibroblast culture consisted of using skin biopsy pieces placed in a tissue culture flask. This is then covered with a thin layer of medium. The media is changed periodically until the fibroblasts migrate

out of the biopsy and divide to cover the bottom of the flask with a confluent monolayer of cells. This method of fibroblast explant culture has the advantage over enzymatic processes in that there is reproducibility of the technique from small amounts of tissue, it is technically a simpler approach versus culture with enzymes and the effect on cells(31). Enzymatic treatments have the problems of over digestion, reduction of cell viability and subsequent poor attachment of cells when not handled properly. There are no recommendations in the literature on the size that the pieces need to be, some groups have used a 4mm human skin biopsy which presumably reflects a common size of skin punch biopsies that are used in the clinic for diagnostic purposes(31).

1.3 The clinical need for skin replacement

Historically a large portion of reconstructive surgery consists of autologous tissue rearrangement to fill defects most commonly involving soft tissues and skin, singularly or in combination. The advancement in the knowledge of the vascular anatomy of the skin and the evolution of perforator flaps have decreased the morbidity that was associated with the original concept of robbing Peter to pay Paul. Here, skin and subcutaneous tissue or muscle if required can be isolated on its vascular supply, thereby ensuring its viability. This refinement has led to only taking what only is required, which results in less

donor morbidity. For example, the workhorse abdominal flap has seen an evolution from using all of the muscle of the abdominal wall which lead to hernias to now only using the skin and fat which has a quicker recovery and less abdominal wall insult. Additionally, industry has weighed in on this evolution and produced better equipment for the microsurgical techniques that are required. As a result, we are now in the era where reconstruction using autologous tissue is at an all-time high, with improving results and lower donor co-morbidities. Despite this competition tissue engineered skin substitutes will have a clinical need. The most obvious areas are sudden large areas of skin loss due to burns, trauma or infection (necrotising fasciitis). Apart from a size limitation, autologous tissue transfer requires optimal conditions for success for example the success of a skin graft is dependent on a non-infected, well vascularised wound bed and is not ideal for coverage of bare tendon or bone devoid of vascularized periosteium or paratenon. Additionally, recipient sites with significant zones of injury can result in unsuitable vessels for microvascular anastomosis. In these situations, biological tissue engineered skin substitutes can be a valuable source of skin cover for the patient.

1.4 Approaches to Skin Reconstruction

1.4.1 Introduction

The reconstructive ladder is one of the first principles learnt in Plastic Surgery with respect to skin wound reconstruction and has been passed down through generations of surgeons (Figure 1.3). Although it has evolved overtime its message remains the same: a logical progression of the surgical approaches that can be utilized in wound healing. The earliest descriptions start with healing via secondary intention (leaving a wound to granulate and ultimately heal with simple dressings), followed by direct closure with sutures, skin grafts and flaps. A flap is the transfer of tissue on the basis of its vascular anatomy from one part of the body to another. It can consist of almost any tissue type, the most common ones being skin, skin and fascia, skin and muscle or skin, muscle and bone. They have evolved significantly over the years in parallel with the increasing knowledge of the intricate vasculature to the skin. Vascularization is a key principle in plastic surgery as tissue reconstruction, be it autologous or from other sources, survives by the establishment of a good blood supply in a timely fashion. As a result the reconstructive ladder has been modified throughout the years to reflect the advances in technology that the speciality has adopted for example advancement in microscope technology that aids free

tissue transfer(9). We are now choosing the most appropriate form of reconstruction for the patient, given the anatomy of the wound and physiology of the patient and can use techniques from any level eg free tissue transfer is a common first choice technique for lower limb trauma defects as opposed to the simplest option before moving to the next stage. The reconstructive ladder however despite its many modifications has not reflected the advances in wound healing, specifically the development and widespread use of dermal matrices. Dermal matrices are dermal substitutes that are FDA approved for wound cover. Furthermore, the potential contribution that tissue engineering of skin and other tissues can make to reconstruction cannot be ignored. The principle being to use biodegradable, synthetic polymers as scaffolds to aid tissue regeneration. Over time these products degrade at a controlled rate and leave behind the patient's own tissue. Therefore, we propose another addition to this reconstructive ladder: biomaterials. This can be thought of as biological (consisting of components of human or animal origin) or synthetic. This will facilitate incorporation of these products as routine in the clinical environment thereby increasing the choice that is given to patients with regard to wound reconstruction, and at the same time embracing the advances in the evolving field of tissue engineering.
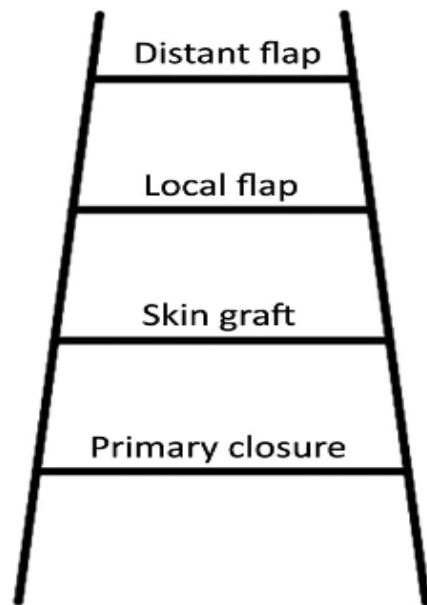
Figure 1.3: *Reconstructive Approaches in Plastic Surgery. A: An early version of the reconstructive ladder. Reproduced with permission from Elsevier. Janis JE, Kwon RK, Attinger CE. The new reconstructive ladder: modifications to the traditional model. Plast Reconstr Surg. 2011 Jan; 127 Suppl 1:205S-212S*

1.4.2 Current Clinical Approach to Reconstruction in Burns

A burn injury depending on the size is one of the very few situations where a patient can lose a lot of skin in a short space of time to the extent that wound coverage becomes a lifesaving procedure after the patient has been resuscitated. Other situations where this can occur are necrotizing fasciitis and Steven Johnsons Syndrome. The decision of how to reconstruct a burn wound in the acute setting is centred on the size of the affected area, the depth of the

burn and patient factors like underlying physiology and co-morbidities. In the chronic setting reconstructions are functional addressing scar contractures that result from healing of full thickness burns or the effect of using of split thickness skin grafts. Historically burn wounds were medically managed with topical therapies applied to the burn eschar(32). In partial thickness burns this worked as, regeneration occurs from the dermal appendages that are left behand. In full thickness burns, once the eschar separates from the underlying wound bed there is was a bed of vascularized granulation tissue suitable for subsequent grafting. This posed morbidities such as prolonged wound healing time, pain, hypertrophic scarring and contractures. The only advantage here was that there was a clearer ability to distinguish between viable skin that those areas that would require grafting. This decision can be challenging in the early stages of a burn as the appearance evolves based on the first aid received, depth of burn and level of fluid resuscitation performed if applicable. Early excision and skin grafting of smaller burns was described first in 1891 by Lusgarten(33). This principle was increasingly adopted by other groups with variable outcomes(34)'(35). It was only in 1981 when Janzekovic reported good surgical outcomes that it became part of the routine practice amongst surgeons worldwide(36). With any burn in the acute setting the immediate management is centred on first aid, assessment and fluid resuscitation only if the burn is more

than 10% total body surface area (TBSA) in a child and 15% TBSA in an adult. Once the patient is stable, the next step is to decide the overall management of the burn. This usually fits into: immediate excision (obvious full thickness based on clinical assessment and mechanism of injury), initial conservative management using dressings under close supervision with a view to possible excision within two weeks (mixed depth burns) and conservative management (partial thickness burns). With regards to surgical management, the approach taken is dependent on the size of the burn and the aim of the excision. In burns that are <20% TBSA, excision occurs on planned burns trauma lists. These burns are often reconstructed with autologous split thickness grafts or dermal regenerative templates depending on the site of the wound. Larger burns (>20% to 50% TBSA) undergo as much excision as possible and reconstruction with widely meshed split skin graft or in some cases cadaveric skin (as will be discussed subsequently) if there is insufficient autologous skin graft to be used. These patients often require multiple visits to theatre as not all areas maybe excised at the same time depending on the availability of autologous skin. With larger burns (TBSA >50%), the aim of excision shifts to survival.

The principle here is that by removing large quantities of eschar, there is a reduction in bacteraemia, endotoxin production and release of inflammatory

mediators. This would decrease the chances of sepsis and multi-organ failure which are the leading cause of death in major burn injuries(37).

The amount of skin excised in a session is guided by the evolving physiology of the patient during the procedure. Furthermore, with larger burns that are predominantly full thickness, the excision changes from tangential (layer by layer excision of non -viable skin until healthy subcutaneous tissue is seen that is graftable) to fascial. Fascial excision, where all the tissue (skin subcutaneous fat) is removed en block, has the advantage of being quicker with less blood loss than tangential excision. The amount of skin excised and the type of excision undertaken influences the reconstructive procedure and ultimately the outcome. The gold standard in large burns is resurfacing as much as possible with autologous split thickness skin grafting. It is often meshed, which means that it is passed through a machine that makes even perforations throughout the skin sheet to increase its surface area and ability to contour around curves (looks like a net). The mesh ratio can be increased to amplify the yield however outcomes such as delayed wound healing, pain and contracture increase exponentially. Commonly used ratios are 1:2, 1:4 and 1:6 for major burns. Another option in this setting is the use of cadaver skin. This provides an immediate barrier that prevents evaporative and thermal losses and allows the patient to stabilize, regenerate used donor sites. A return to theatre about 3

weeks later is undertaken to remove the cadaver skin and graft onto the underlying wound bed.

In the long term setting surgical intervention is centred on scar revision and contracture release. Early excision is proposed as a technique to circumvent this and the Burns multidisciplinary team is established to optimize patient care. This can have a profound effect on scar outcome; however, there will be some patients that present with hypertrophic, pigment -variable scars and contractures in reconstructed areas.

Contractures usually arise because of a deficit of skin and surgery is designed to replace that deficit. The two main decision making points at this stage rely on whether local skin is suitable. Skin and subcutaneous fat can be imported either from a distant area in the body using regional pedicled flaps (tissue containing normal skin and fat, free of contracture but comes with its own blood supply) or free tissue transfer (skin and fat free of contracture but has to establish its own blood supply via microanastamosis of veins and arteries at the site of the skin deficit). Additionally, skin can be imported by using dermal regenerative templates. The general consensus is that, if possible full thickness grafts or flaps are the best options if available. This is because split skin grafts and dermal substitutes have a significant amount of secondary contracture compared to full

thickness grafts and flaps. Furthermore, they require more aftercare (splints, physiotherapy and pressure garment use) and revisional surgery later on.

Full thickness grafts are used for moderate sized defects following contracture release. Large donor sites can be obtained provided that these are burn free. For example, the abdomen can provide large quantities of skin (depends on patient size and abdominal excess) via various transverse lower abdominal incisions and also performing an abdominoplasty (removal of excess skin and fat from the abdomen en bloc). Other sites like post auricular and supraclavicular skin can provide small amounts of skin (<0.5% TBSA) but good colour matching to the head and neck area. As a rule, any site of skin laxity that can be closed directly can be the donor site of the full thickness graft and can utilize aesthetic techniques to obtain skin. These include offering the patient a breast reduction or a mastopexy (breast lift) (females only and males with gynecomastia or enlarged breasts). These procedures often result in large quantities of skin and subcutaneous fat that are not needed by the patient. Therefore, full thickness grafts in these settings are large, require careful handling and removal of as much subcutaneous fat as possible to ensure successful graft take.

Flaps are another source of excellent skin and subcutaneous tissue to resurface scars and contractures. Local flaps if suitable tissue exists are the ideal method of reconstruction as it provides good colour and texture match and little

aftercare. However, these are limited depending on the site and size of the skin defect resulting from the contracture release. Furthermore, even if suitable tissue exists they maybe bulky as flaps may have variable amounts of subcutaneous tissue compared to the wound bed of the contracture release.

Free tissue transfer has become quite popular as a method of reconstruction for contracture release due to advances in microscope and equipment technology and greater understanding about the vascular anatomy of the skin. Free flaps however are reserved for complex and difficult areas like the head and neck area and the upper and lower limb. The recipient vessels must be free of burn injury, as the flap would simply fail which would lead to significant morbidity for the patient. Colour and contour matching are challenging as in principle the free flap transfer dictates that it can be taken from any suitable area in the body distant to the skin deficit site.

1.4.3 Skin Grafting

1.4.3.1 History

Skin grafting, one of the oldest and most common techniques used in skin reconstruction dates back to 1500 BC (38). It was modified and gained popularity in Europe during the late 1800s. Pinch grafting developed by Reverdin involved

placing 1mm square pieces of epidermis on the granulating tissues leading to rapid re-epithelization of wounds(39). This technique was associated with significant contracture and therefore led to the use of strips of tissue placed close together containing both epidermis and dermis resulting in better outcomes in terms of time to wound healing and scar contracture. This was the main method of skin grafting used in Europe until the 1940s(40). At this time, the evolution of how skin grafts needed to be optimized for successful take was investigated. It was determined at this time that grafts with thinner dermis are better suited for coverage of granulating wound bases as opposed to recipient areas that subjected to trauma and where it was necessary to avoid contractures like over the flexor surface of a joint. Grafts were therefore classified according to their dermal thickness (Table1)(41). This classification on dermal thickness for split thickness grafts was discussed in one of the investigative chapters in this thesis, as we wanted to determine the effect of cellular migration from the cut edges of the split thickness skin explants. Following this, surgeons wanted to standardize the method of harvesting grafts so as to get a fixed proportion of dermis needed for a particular application. This lead to the evolution of the various instruments used to harvest a graft. In the late 19th and 20th centuries the Humby knife was used however dermal thickness was still not reproducible using this technique(42). The dermatome was then developed, which was

initially manual, relying on a rotating blade fixed to a drum. This harvested skin of uniform thickness however the graft tended to separate from the drum(43). It was then refined and the air powered dermatome was developed, which is currently the most widely adopted instrument in harvesting a split thickness graft containing a consistent amount of dermis(44).

After refinements were made in harvesting grafts, there was focus on the best method to expand them. It was known that grafts could be expanded to cover large areas without compromising re-epithelization if done in the correct ratio. Furthermore, expansion revolutionized the treatment of major burns, as less donor sites were needed to treat a given surface area of burn. Additionally, expanded grafts contour curves easier and the spaces in between the grafts allow for the extrusion of blood and tissue fluid that can collect under an unexpanded graft lifting it from the wound bed and resulting in a failed application.

Category	Standardized thickness inch; mm
Thiersch (epithelial layer only- rarely achieved)	0.008-0.010; 0.2-0.025
STSGs	0.012-0.106; 0.3-0.4
Three quarter skin grafts	0.018-0.022; 0.45-0.55
Full thickness skin grafts	0.030-0.038; 0.75-0.95

Table 1: *Initial classifications of thickness of skin grafts (41).*

1.4.3.2 Meek Grafting

In 1958, Meek an American Surgeon proposed the development of a dermatome that allowed a 1 inch square of skin to be cut into 256 square pieces(45). Meek theorized that wound re-epithelization occurred from the cut edges of the small squares of split thickness skin grafts therefore increasing these edges by increasing the surface area resulted more rapid wound re-epithelization rather than using one large square. The graft squares were placed on prefolded gauze to achieve an even distribution. This technique had several advantages in that there were high rates of successful take and the expansion ratio was true to the number desired (eg a 1:4 ratio was actually 1:4) as the squares were totally separated from each other whereas in meshed/net grafts the ratio is user dependent on the amount of stretch applied. Moreover, small graft remnants are utilized and there is no wastage. As a result there have been successful reports of its use in the management of major burns(46) however it did not gain popularity in Burn Units in the Western world as the dermatome itself was expensive at that time, but more so it was labour intensive as each skin graft square needed to be placed dermis side down (Figure 1.4a). This technique is not practical for large burns where time is a limiting factor in the

initial excision and reconstruction. Over the years many modifications to the

Meek technique have been explored (Figure 1.4b) and good outcomes have

been reported in the literature with regards to time to wound re-epithelization

leading to a resurgence in its usage (Figure 1.5)(47),(48),(49). This principle of

keratinocyte migration in Meek Grafting can be used to populate the skin

substitute with a patient's own cells via small split thickness skin explants

thereby negating the need to use a split skin graft and laboratory-expanded

cells, which in itself is associated with a high cost, technical expertise and the

use of a clean room environment which is highly regulated and requires highly

trained staff. Hopefully this will give the proposed construct the flexibility that

is required in a dynamic and unpredictable clinical environment.
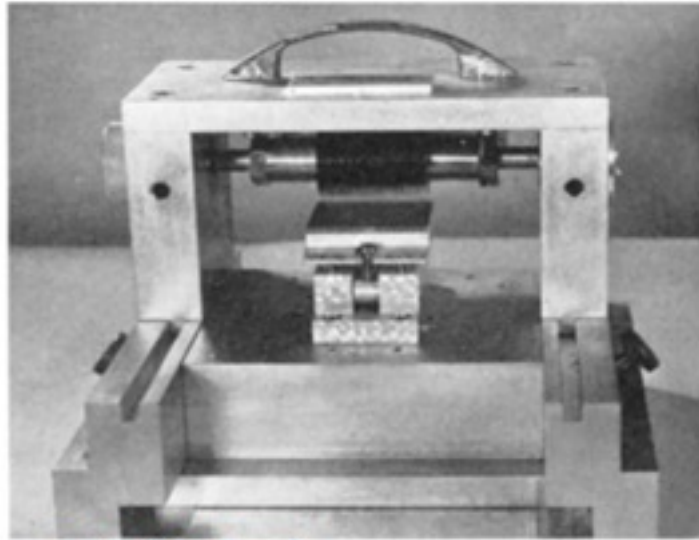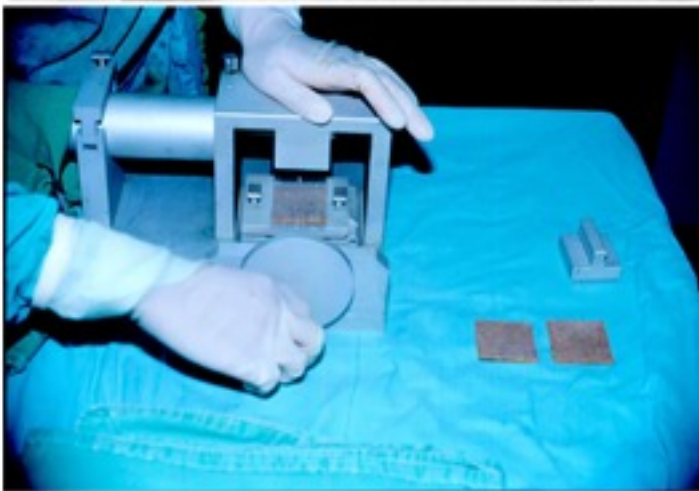
Figure 1.4: *Meek Dermatomes.* *A: Original version reproduced with Permission from Elsevier. MEEK CP. Successful microdermagrafting using the Meek-Wall microdermatome. Am J Surg. 1958 Oct; 96(4): 557-8. B: Modified Meek Dermatome in current use. Reproduced with permission from Elsevier. Hsieh CS, Schuong JY, Huang WS, Huang TT. Five years' experience of the modified Meek technique in the management of extensive burns. Burns. 2008 May; 34(3): 350-4.*
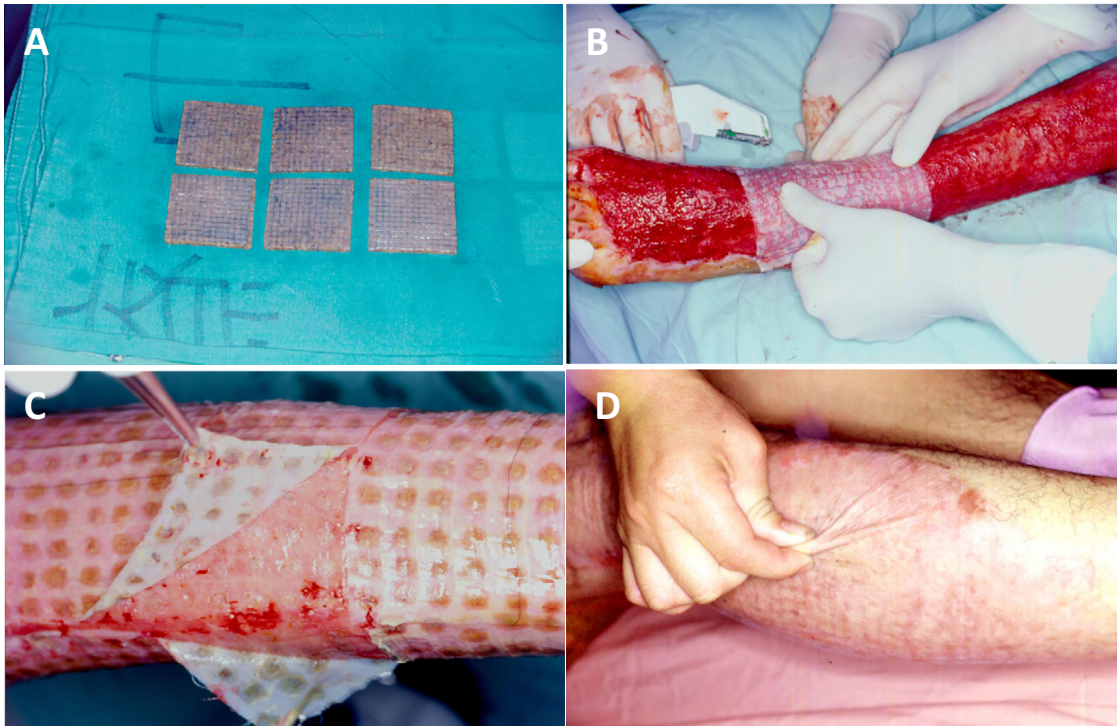
Figure 1.5: *Method of modified meek grafting in practice. A: Grafts harvested as small postage stamped squares, laid onto gauze; B: Application of the graft onto a debrided leg wound; C: Graft check at 10 days; D Long term outcome of healed wound that was treated with Meek Grafting. Reproduced with permission from Elsevier. Hsieh CS, Schuong JY, Huang WS, Huang TT. Five years' experience of the modified Meek technique in the management of extensive burns. Burns. 2008 May; 34(3): 350-4.*

1.4.3.3 Meshed Grafting

The main method for graft expansion that is the most widely accepted and currently used in the United Kingdom was developed by Tanner in 1964(50). This is known as a meshed graft and can achieve in theory ratios of expansion from 1:1.5 to 1:6. They work well because they have high rates of take compared to unmeshed grafts, they allow for drainage and are more flexible when being

draped over areas with contours. The major disadvantage however is that they leave behind a scar that is well healed with a net like pattern. This makes it significantly cosmetically inferior to a sheet (not meshed) graft. Therefore, many patients opt for serial excision of the meshed graft once healed if possible, which leaves them with a fine linear scar.

1.5   A review of skin substitutes

1.5.1 Properties of an ideal skin substitute and current uptake of skin substitutes in the United Kingdom.

Any model, which aims to replace skin, should replicate not only the anatomy but also the physiology of the organ. In this respect skin substitutes need to possess essential properties for them to be used. All substitutes need to be nontoxic, biocompatible, non-immunogenic and pathogen free. Most biological skin substitutes have products of animal origin eg bovine and porcine. Although disease transmission is rare, the advances in synthetic biocompatible materials should mean that we do not have to rely on animal based products. Efficiency of a skin substitute means that they should work and the outcome should be predictable within the realms of obvious known complications. This allows the surgeon to accurately inform the patient about the product in order to gain

informed consent. Other desirable properties include reasonable cost, long shelf life, being readily available and ease of handling. Furthermore, a product being off the shelf that is easy to use are also desirable properties. Current biomaterials are not infection resistant and are actually more susceptible to infection than autologous skin. A study by Bargues et al(51), demonstrated that the popularly used Integra, was associated with a 42% infection rate in their series which was higher than others (52). They reasoned that delayed application is a likely causative factor as the most common organisms cultured were Staphylococcus Aureus and Pseudomonas (51). Although this can happen with autologous skin grafting, the studies performed are not methodically robust to definitively associate the use of Integra with an increased infection rate with Integra being the causative factor. Furthermore, as infection post skin coverage is a multifactorial process, it is challenging to define which aspect of the material (properties of the material itself or its application) are responsible for this.

Another area that is lacking in the current stock of skin substitutes is the paucity of composite skin substitutes to offset the need to use either laboratory expanded cells or thin split skin grafts on a dermal scaffold. The most commonly used biological scaffolds are dermal in origin which then needs to be populated with epithelial cells. This often requires planning and another operation. As a

result, it was felt that skin substitutes consisting of dermal and epidermal components in one sitting, would be a more time effective approach to augment wound healing. An example of this is Apligraft (discussed below), which is marketed for smaller wounds and limb ulcers.

Some skin substitutes require a patient biopsy to culture and expand cells that are then laid onto a scaffold or carrier. This means that the product needs to be used within a very defined and narrow timescale, which may not be possible if the patient is unfit for theatre at that point in time. Furthermore, purely dermal analogues still require a split skin graft albeit a thinner one but this requires another trip to theatre. Therefore, the ideal material would be safe, effective in replacing skin and have reproducible and predictable behaviour when placed onto a wound bed. Ideally it should be free of animal products and not require a clean room in a laboratory for expansion of cells as this increases expense and expertise required for production of the skin.

1.5.2 Skin Substitutes in Current Clinical Use

This has been part of the armamentarium of the reconstructive surgeon for over three decades. They can be divided into either temporary or permanent and can be further classified into epidermal, dermal and composite replacements. There

are a few staples that have become a permanent feature of theatre stock lists, despite their limitations, as their results are reproducible and undoubtedly beneficial to the patient. A survey conducted by Dunne et al in 2015 across 14 Burns in the UK, Australia and New Zealand, revealed that in the Paediatric population, Biobrane (a temporary skin dressing, discussed subsequently in the chapter) is a popular temporary dressing of choice (86%). Cadaveric skin, two stage Integra and Matriderm were also commonly used in this population for resurfacing larger TBSA burns. For adults; Cadaveric skin, two stage Integra and Matriderm were popular as well, with the uptake of Biobrane (64%) less in this population(53).

1.5.3 Temporary Skin Substitutes

Cadaveric Skin

One of the most commonly used temporary skin substitute is cadaveric skin. The first documented use of cadaveric skin in covering burn wounds was in 1881 by Girdner (54). Cadaveric skin is currently used as a temporary dressing on a freshly excised burn wound. It prevents evaporative losses, improves thermoregulation, decreases bacterial load in contaminated wounds and may provide dermal regeneration elements, which can persist once it is removed.

There are two main types of cadaveric skin allografts based on the method of processing and storage: cryopreserved and glycerol preserved(55). In the context of large total body surface area burn excision in the acute phase, it provides a stimulus for the release of growth factors and cytokines in the wound bed. It also increases the vascularity with enhanced capillary ingrowth of the wound bed. The take rate of skin grafts following application of cadaveric skin to prepare the wound bed can be as high as 88.4%(55). It can also be used as a definitive dressing in partial thickness burns, which is associated with less painful dressing changes. This is particularly important in the paediatric population as burns dressing changes are associated with physical and psychological distress. Its use however in this population has been superseded by Biobrane which is popularly used in partial thickness burns. The use of allograft cadaveric skin is associated with a decrease in infection, fluid and heat loss, and pain as well as optimal wound bed preparation prior to application of autograft(56).

Its main limitation revolves around donors and the organization and funding of tissue banks that prepare the skin. Although allograft is very much used in many plastic surgery and burns units across the United Kingdom, there are still challenges with respect to its supply (57). The United Kingdom has centralised their supply of allograft in light of the decreasing requirements to cover very

large TBSA burns and the increasing legal requirements that need to be met in order to store human tissue on a long term basis. As a result xenografts most commonly porcine eg E-Z Derm® are often used as temporary dressings, which have the advantage of low cost, prolonged shelf life, availability and convenience particularly when allograft is not available and coverage is only required for a short time (58). Furthermore tissue harvesting from the deceased is a relatively a new concept in many developing countries as some religious beliefs can view this as mutilation of the body(59). Furthermore, despite rigorous screening, there remains the risk of prion disease transmission.

Having said this, the Euro skin bank, established by that name in 1992, formally the Dutch National skin bank, represents an efficient practice of harvesting, storing and providing glycerol preserved cadaveric skin(60). Glycerol is advantageous as it preserves the architecture of the matrix proteins, has antibacterial and antiviral properties and it is thought to diminish antigenicity(61). Organization is the key to the skin bank's success as it is hosted in its own facility staffed with an independent team of technicians, research, managerial and medical personnel. Additionally, they are in close proximity to a hospital that treats major burns which allows for a feedback mechanism on quality control and assurance(60). As a result, the uptake and use of glycerol preserved cadaveric skin became so popular in the early 1990s, that the

organization now receives skin for preservation and storage from other European Countries to meet demand. Critically, they have established their own research division supported financially by the Dutch burns Foundation to obtain a better understanding of the properties of glycerol preserved skin in wound healing. The centralisation of the skin bank in this way not only allows an amalgamation of expertise but benefits from an independent administration network to ensure that their needs are met to upkeep with their demand.

Xenografts

The first xenograft to be used as a human skin substitute came from sheep in 1880 (56). Porcine products are most widely used as a substitute for allograft due to the similarities with human skin. These include comparable epidermal thickness, turnover and migration, hair distribution, dermal elastin structure and collagen macrostructure(62)(63).

There are various preparations that are available for clinical use and the method of preparation influences the structure and contents of the product that is ultimately placed onto the patient. These include fresh, fresh frozen, lyophilized, irradiated and aldehyde cross-linked(64)(65). The fresh, fresh frozen, lyophilized and irradiated forms contain both epidermal and dermal components, however the aldehyde preparation results in removal of the epidermis and dermal

appendages leaving behind a sterile dermal acellular matrix that can be used either side down and stored at room temperature(66)(67). Xenograft skin substitutes are most commonly used as a temporary dressing to aid wound bed preparation prior to autograft application in full thickness burns, as a definitive dressing in partial thickness burns and donor sites and in the treatment of toxic epidermal necrolysis(68). It has been developed in an attempt to meet the need to have a constant supply of allograft. One such preparation that is in clinical use is EZ Derm® (Molnlycke Health Care, US, LLC, Norcross, GA). This is an aldehyde cross linked porcine acellular dermis that is available for off the shelf use as a definitive dressing in partial thickness burns(69). It has been shown to reduce the pain associated with dressing changes thereby reducing the need for them to be done under general anaesthetic. It can also be used in patients who are not physiologically fit for extensive skin reconstruction or in large burns with an autograft shortage. It acts as a definitive barrier against evaporative skin losses and micro-organisms temporarily, whilst donor sites are regenerating or the patient is being physiologically optimized for further surgery. Porcine products form fibrin-collagen bonds with the underlying wound bed that allows the dressing to adhere readily. There is no vascularization or take in xenografts, unlike allograft which is beneficial in its indicated use as a temporary

dressing(58). In these circumstances the dressing is left in place until it separates from the underlying healing skin (in the case of partial thickness burns).

Biobrane (UDL Laboratories, Rockford, IL)

Biobrane was developed in 1980 by Tavis(70). It has a bilaminar structure consisting of a woven nylon membrane coated with silicone to which collagen is chemically bound. It aims to prevent evaporative fluid losses and development of infection when used as a dressing in superficial partial thickness burns. Its physical macrostructure and collagen binding attracts fibrin and ingrowth of fibroblasts from the underlying wound bed, so that the product adheres tightly to it. This is translated in its use, as once Biobrane is applied to the underlying wound it is trimmed away as it separates from the underlying regenerating skin as opposed to being peeled off the wound during a dressing change. As a result it is advocated to provide painless dressing changes which reduces psychological and physical distress more so in children avoiding the need for another general anaesthetic(71). It is also used as a dressing in toxic epidermal necrolysis and for donor sites from autologous split skin grafting, especially when dealing with the larger TBSA burns(72).

The major limitation with the use of Biobrane is the complication of infection. It is not advocated for use on an infected or potentially infected surfaces or an

eschar. It should also not be used on deeper burns, where the dermis is affected.

This is because fluid collections form between the wound bed and the biobrane

which can go onto become infected. There have been reports of life threatening

Staphylococcal toxic shock syndrome after Biobrane use, which is a major

morbidity especially in the paediatric population where this product is

commonly used (73)(74). However careful monitoring and continuous

assessment can avoid this for example performing a dressing change within 24

hours post application, venting the Biobrane above any areas of fluid collections

and removal of any areas that are not adherent(75).

1.5.4 Permanent Skin Substitutes: Epidermal and Dermal Replacements.

Epidermal replacements

The main epidermal substitute that is clinically in use is cultured epithelial

autografts (CEA)(76). These are used primarily as an adjunct to split thickness

grafts rather than on its own, as it has been reported to provide suboptimal

outcomes at a significant expense(77). Clinical take has been reported at

45%(77). They can be used over widely meshed grafts or donor sites to expedite

healing. They can also be placed on dermal scaffolds eg Integra or dermal

allograft for partial thickness defects where some residual dermis will allow for

attachment of keratinocytes. The real difficulty occurs in the timing of the production of CEA sheets to meet that of the patient as it takes on average 12 days to produce sheets with confluent cells before they can be clinically used. Furthermore, these sheets are very fragile and must be handled with utmost care to prevent fragmentation. New methods have since emerged to deliver these cells to patients, in the form of specialized carrier dressings (78)and suspension sprays(79).

Cell suspensions contain basal keratinocytes, melanocytes and papillary fibroblasts. They avoid the use of Dispase, an enzyme used to release the epidermal sheets from culture, which removes surface proteins from keratinocytes, thereby reducing its adhesive potential(80). Furthermore sub-confluent cells can be used which reduces the laboratory processing time and it is thought that keratinocytes in this form would have superior proliferative potential(79).

Carrier dressings for sheets of keratinocytes have also been developed to aid transfer of sheets with minimal damage to cells. With respect to its clinical uptake MySkin (CellTran Ltd, Sheffield, UK) has been available as a product in the UK since 2004. From 2004 to the present time, the company has worked

with surgeons to provide laboratory expanded skin cells for 192 patients at 23 treatment centres in the UK. 18 of these centres have used MySkin for at least 3 patients and 5 centres have treated more than 10 patients with MySkin. In terms of living cells this is the most commonly and frequently used project in routine NHS use in the UK to the best of our knowledge.

The proposed benefit of cultured keratinocytes is the potential to increase wound epithelization due to the presence of basal keratinocytes and transplantation of melanocytes which aims to maintain the colour of the patient's skin as the biopsy to source the autologous cells is usually taken just adjacent to the defect(81). Multiple studies have investigated the use of CEA either alone or in combination with other products like Integra. When CEA on their own are applied to excised burn wounds, take can be inconsistent. For example one case series reported an overall take of 80% for burns with a mean of 66% TBSA(82). Furthermore, for burns larger than 80% TBSA required at least two applications of CEA. Other improved outcomes included reducing donor site scarring when the CEA was applied to these areas(83). One of the fundamental problems of CEA, is its fragility both at the time of application and after take occurs. The most commonly used application of CEA occurs in patients with burn injuries that are medium to large in nature (20-30% TBSA depending on the size of the patient)

and can range from partial thickness in which some of the dermal regenerative elements are left behind to full thickness burns that can extend beyond the dermis. It is no surprise that there are reports of better CEA take in partial thickness burns. However, in large TBSA full thickness injuries, it has been reported that there was 60% reduced adherence of the CEA up to 6 months post application whereby the sheets were dislodged with minimal shearing forces and these areas simply blistered and sloughed off. (84). CEA provides epidermal cover, skin loss in large burns, of which this application is most used, can be at times mixed depth or full thickness. In these cases, the dermal regenerative elements located at the base of the pilosebaceous unit have been destroyed and as a result, the dermis is not regenerated. CEA applied to these wounds or onto subcutaneous fat or fascia, will not take. Studies have indicated that there is a lack of dermal attachment by way of anchoring fibrils between the applied CEA and the underlying tissue some of which will be native dermis(85). Other reported challenges with the use of CEA include infection, blistering and cost considerations.

The time, expense and expertise needed to culture keratinocytes in the lab as part of the application process of CEAs led to the development of an on the spot cell delivery system called ReCell® (Avital Medical, Valencia, CA, USA).

A small sample of split thickness skin is obtained close to the area to be reconstructed and added to a small container containing an enzyme (porcine origin) and heated for about 15- 30 minutes to and added to a buffer solution to obtain a suspension containing keratinocytes, melanocytes, fibroblasts and Langerhans cells. The suspension is then delivered either to a debrided wound bed (with dermal components present- known as mixed depth) or used on top of widely meshed autograft in full thickness wounds using a spray applicator or poured directly onto the site.

The technique is designed to be carried out in theatre, by clinicians trained in the procedure. The key point in this application is that it does not result in true cellular expansion by numbers- it simply releases the cells from the extracellular matrix from the skin sample and laid over an expansion ratio of 80:1 in an attempt to aid epidermal regeneration and re-epithelization. It has also been reported to treat conditions like rhinophyma and congenital melanocytic naevi where in both these cases, post debridement, the dermal components are present (86)(87). A study by Holmes et al in 2018 comparing the use of ReCell combined with widely meshed STS vs STGs alone revealed that donor areas harvested combined with Re Cell, were superior in terms of healing at weeks 1 and 2 post op respectively compared to STSGs alone(88). This was to be expected as used thinner STSGs were used with the ReCell group than the

control versus the same thickness and evaluation of time to healing with addition of Recell which was either way found to be not difference between the two groups. Furthermore In 2007 Gravante et al compared ReCell versus skin grafting for deep partial thickness burns and found no significant differences in time to complete epithelization, postoperative pain and contractures (89). Procedure time was found to be significantly longer in the cases that used ReCell versus skin grafting. Studies have also investigated the use of ReCell in combination with split skin grafting and Biobrane(90) however there was not enough statistical analysis to accurately determine whether these combinations conferred a worthwhile benefit. The National Institute of Clinical Excellence (NICE) which provides recommendations to English health care providers on use of medicines and products has not supported routine adoption of the use of ReCell due to there being insufficient evidence of its cost vs benefit analysis. They recommended that further robust studies were needed to define its role in the surgical treatment of deep to full thickness burns and its use is available on an NHS Trust policy basis. Accordingly at the time of writing the true uptake of ReCell in the UK is not known (91).

Dermal Analogues

Integra (LifeScience Corporation, Plainsboro, N.J.)

With respect to dermal replacement materials, Integra is currently the most widely accepted synthetic dermal substitute that is used across many areas of skin reconstruction (92)'(93). It is a bilaminar structure consisting of cross-linked bovine collagen and glycosaminoglycan on one side with a silicone membrane on the other. The pore size in the biological layer ranges from 70-200 μm to allow migration of fibroblasts and endothelial cells, which is needed for integration(94). The product is applied with the biological layer facing the wound bed, secured at the edges with sutures or glue and dressed to facilitate regular inspections. The collagen layer then bio integrates with the wound bed to form a vascular neodermis. This takes about 3-6 weeks. At this point the patient is taken back to theatre and a thin split skin graft is applied.

Integra has been successful in the clinical environment as it is readily available (can be stored in the hospital and used when and as required), easy to apply, has a predictable course that is identifiable, for example the ability to determine take versus failure or infection. Trials have reported superior cosmetic outcomes of the donor site (more thinly harvested than autograft alone), with faster healing times(95).The longest published series using Integra reported benefits such as over 80% take rates with no severe hypertrophic scar formation and excellent function where it has been used over involved joints(96). However, these trials are flawed by the fact that outcome measurements for scar

assessment were subjective and not validated. Interestingly histological studies performed on areas with Integra over a two-year period revealed remodelling of the dermal component over the first month of application with features that looked like papillary and reticular dermis but with no rete ridges(14). As with any product there are areas that can be improved upon. These include the fact that it is most commonly used as a two-stage procedure (single stage Integra is available), requiring a return to theatre and the biological layer contains products of animal origin. Furthermore, it is quite expensive which limits its use in developing countries that may actually need it the most given the higher incidence of larger burns.

Matriderm (MedSkin Solutions Dr Suwelack AG, Billerbeck, Germany)

This is another dermal analogue consisting of single laminar matrix of collagen and elastin. The collagen matrix is isolated from bovine dermis and the elastin is obtained from the bovine nuchal ligament by hydrolysis(97). This is marketed as a single stage dermal template, whereby it is applied to the defect in addition to a very thin split thickness graft in the same operation(98). Matriderm is widely used in skin reconstruction in many areas for example post skin cancer excisional surgery, burns (deep to full thickness) and traumatic wounds. It handles easily and acts as a haemostatic agent once placed on a freshly debrided wound bed.

A thinner than average split thickness graft is then placed on top as a source of epidermal cells. It is thought that the combination of collagen and elastin promotes vascularization quicker and support the ingrowth of cells and vessels. Matriderm is used in the management of burns both in the acute and chronic settings for burn scar contracture reconstruction. Studies have indicated it minimises scar contracture and increases skin elasticity and flexibility when compared to using autologous SSG. Furthermore a thinner split skin graft means a donor site containing much more dermal regenerative elements, so there is less pain and quicker healing at the donor site(99).

Composite Skin Substitutes

One of the most notable translated composite skin substitute material was performed by Boyce and colleagues. This consisted of creating a cultured split skin (CSS) graft in the laboratory and applying it on debrided wounds pre-treated with allograft which optimized vascularization. In later studies they replaced allograft with Integra(100). A biopsy of the patient's skin was taken for cell culture and expansion to create an epidermal layer. The dermal analogue consisted of a bovine collagen-glycosaminoglycan (CAG) substrate. The epidermal cells once expanded were inoculated into the CAG substrate at high

cellular density. These were cultured until keratinocyte stratification and confluence were found to be optimal as checked by light microscopy(101). Patients with > 50% TBSA burns were used in its evaluation and it was compared to the gold standard split thickness skin graft by using both techniques on adjacent sites on the same patients. They reported that areas treated with the cultured skin substitute had superior appearances compared to autologous split skin grafting at five months, due to the lack of hypopigmentation and meshed pattern which are common when autologous split skin grafts are used(102). Furthermore, it was reported that for excessively large full thickness burns (TBSA 88%) complete coverage was achieved using Integra and CSS by 55 days. This technique combined the use of a material developed by Burns Surgeons to provide immediate wound cover with pioneering tissue engineered biomaterials to achieve notable patient outcomes.

This work highlights two key points, firstly that it is possible to use cultured skin in large burns in a relatively timely manner and the importance of achieving preparative wound bed vascularisation. In the absence of a well debrided vascularised wound bed, this or any other technique will not be successful. This technique requires close liaison with an appropriate laboratory that can deliver these cells to the patient in a timely manner which has always been challenging to organize and implement as part of routine clinical practice.

Apligraf (Organogenesis, Canton, MA)

This was previously called Graftskin and is another biological composite skin substitute consisting both of epidermal and dermal components(103). The dermal aspect is mimicked using a scaffold consisting of bovine type I collagen and human derived foreskin neonatal fibroblasts. The epidermal component consists of human foreskin derived neonatal epidermal keratinocytes that are cultured onto the dermal scaffold and allowed to stratify before transferring it onto the patient(104). Thus this is based on allogeneic cells which will not survive long term in patients. It was initially approved by the FDA for the treatment of venous ulcers that failed trial of conventional treatment for one month. It has also been evaluated in other clinical applications such as following Moh's or excisional surgery for cutaneous cancers where it was compared to healing via secondary intention. In this case, it is reported that Apligraf was associated with a less vascular scar and a better cosmetic appearance than those healing by secondary intention(105). Furthermore, when it is used to aid donor site wound healing following grafting of large burns, it is associated with less pain when compared to simple polyurethane films(106). It was also used in the reconstruction of small full thickness burn injuries where it was shown to expedite wound healing(107). The evidence for its successful use is limited to

level IV and V clinical studies, with low patient numbers and no definitive comparison to the clinical gold standard which is an autologous skin graft.

Most of its advocated clinical use lies in the field of venous and diabetic foot ulcers where it has been shown to be a cost effective option in the long term management of these chronic wounds as it offsets the need for continuous dressing and treatment of episodic infections due to its associated significantly shorter healing times when used(108)(109). This product has not gained popularity amongst reconstructive surgeons in the United Kingdom as a routinely used skin substitute(53).

1.6 Electrospun Scaffolds

1.6.1 Introduction

Electrospun scaffolds have been at the forefront of tissue engineering with many uses in healthcare ranging from tissue regeneration to drug delivery systems. Scaffolds have been developed in almost all areas where there is lack of native tissue and current clinical management does not yet yield optimal results for example bone replacement, treatment of cleft lip and palate and skin. Scaffolds can be synthetic or biological. Scaffolds fabricated using electrospinning have a wide variety of biomedical applications due to the

flexibility in mimicking some of the properties of the native tissue. Networks of both micro and nanofibres can be made separately, in layers; they can be templated or bioactive agents eg vitamin C(110) and anti-inflammatories (111)or hormones such as oestradiol (112)can be delivered from the fibres. This results in a range of scaffolds consisting of various orientations of fibre size and diameter that are comparable to the structure of the extracellular matrix of many human tissues.

1.6.2 Technique of Electrospinning

Many tissue-engineering laboratories across the globe have been investigating the use of synthetic electrospun scaffolds as substitutes for native extracellular matrix for decades. The principle relies on a high voltage (e.g. 17000 V) travelling between a syringe driver and a conductive collector to extract thin fibres from dissolved biodegradable polymers. The dissolved polymer is loaded into a needle-tipped syringe (various sizes eg 5,10 or 60 ml) and an earthed target collector is positioned at a set distance from the needle tip. This distance varies for each polymer used and the fibre size required. Application of a high voltage

to the needle tip (eg 17000V) causes the dissolved polymer to charge and form a Taylor cone.

Once the charge of the solution is strong enough to overcome the surface tension and viscoelastic forces of the Taylor cone, the polymer expels in the form of a charged jet. This travels across an air gap whilst simultaneously undergoing solvent evaporation, stretching and thinning. Once the jet comes into contact with the earthed collector, the process is complete, the charges dissipate and networks of fibres are formed(113).

Fibres are randomly orientated or well aligned depending on the rate of rotation of the collector. Randomly orientated fibres are produced when the collector is left stationary i.e. static spinning or spinning at low speed. Well aligned, ordered fibres are achieved using a more rapidly rotating mandrel eg > 500 rpm, with adjustable speeds that can further influence the properties of the fibres. Another commonly used speed was 200 rpm to create the initial trilayer electrospun scaffold architecture(114). The diameter of the fibres can be modified by changing any one of many parameters in the electrospinning set up (Figure 1.6). These fibres form a 3D scaffold onto which cells can proliferate, migrate and function to achieve the spatial organization that is required for it to form a tissue.
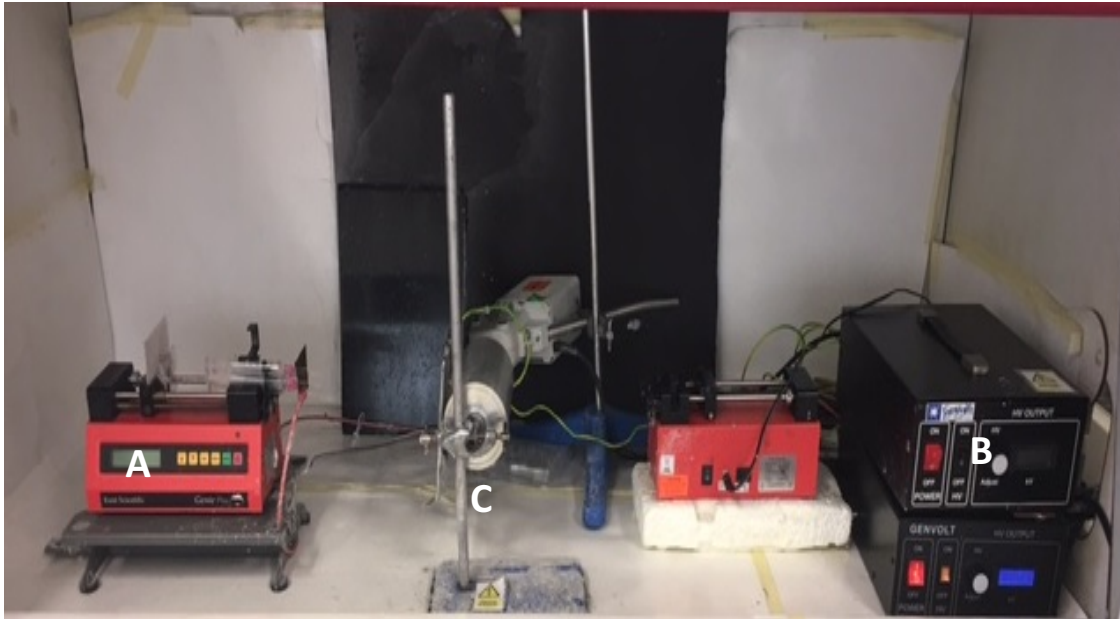
Figure 1.6: *Electrospinning Set Up: A- Syringe Driver, B- Electric Generator, C-Mandrel*

1.6.3 Polymers used in the Electrospinning Process

These can be thought of as chemical compounds that have the ability to assemble in simple structural units to create a 3-dimensional construct with various applications in biological systems(115). Polymers developed for use in biomedical applications can be thought of as natural or synthetic and permanent where they remain in situ or temporary where they degrade over a specific time period. Their use is flexible and variable ranging from structural proteins of the extracellular matrix to matrices with mechanical functions like ligaments, cartilage and skin. Furthermore, they can also serve to augment the function of

existing organ systems eg vascular grafts in occluded arteries. Natural polymers have been in use since the dawn of time eg animal horn, hair, cellulose and silk, however with concerns of immunological reactions and variable batch to batch reproducibility the focus is now on synthetic polymers that have biological capabilities(115).

Man-made biocompatible synthetic polymers are attractive materials for clinical applications. A wide range of chemical and physical properties can be achieved with these compounds to fulfil the structural and mechanical properties that are required for the specific tissue application. Polymers used in biomedical applications are biodegradable and can be designed to stay in the body for as long as it is needed and then disappear without the need for any surgical intervention to physically remove them. Furthermore, the breakdown products are non-toxic and excreted in a predictable fashion. These were initially developed for orthopaedic applications like internal fixation of bone (116), but have now extended to vascular stents(117),(118) and drug delivery systems(119).

Synthetic, hydrolytically degrading polymers are preferred for implants or drug release systems, as they are independent of the patient or implantation site (120).

Biodegradable polyesters are just one category of polymers employed in tissue-engineered bioproducts. The most common ones consist of polyesters of small aliphatic glycolic acid (PGA), lactic acid (PLLA) and polycaprolactone (PCL)(121). PGA degrades through hydrolysis of its ester bonds and is eliminated ultimately as water and carbon dioxide. It loses its strength in vitro within 4 weeks and is completely absorbed within 6 months. As a result, it is often combined with other polymers like PLLA to slow its rate of degradation, which can be tailored to the specific tissue target(122). Another polymer in this group polydioxanone (PDS) is a commonly used absorbable suture in current surgical practice(123).

Polylactic acid (PLA) and polyglycolic acid polymers are often chosen for dermal replacement as their mechanical properties and rates of degradation match that which is needed for skin wound repair. Blackwood et al established that a copolymer of poly (D, L) lactide-co-glycolide (PGLA) at a 75:25 ratio of PLA to PGA was appropriate for use in human dermal replacement as it supported the migration and proliferation of keratinocytes and fibroblasts and facilitated collagen production and deposition. Additionally it did not undergo necrosis or encapsulation and degraded by 50% within three months when implanted in rats (124).This is thought to be physiologically desirable as by the three-month mark, the wound would ideally be at the stage of remodelling, where the

patient's own deposited collagen III is replaced with collagen I thus negating the need for mechanical support or presence of the scaffold.

1.6.4 Mechanical Properties of electrospun scaffolds

In order to use electrospun scaffolds in biomedical applications, their mechanical properties need to be assessed to determine their suitability. The strength and deformability of the fibres must match as closely as possible the tissue one is trying to replace. This is especially important for example in the manufacturing of biosynthetic vascular grafts, which are proposed for use in atherosclerotic arteries. Any material that aims to replace a vessel, must match its physical and mechanical properties so as to avoid the disastrous consequences of graft failure e.g. aneurysm formation if the walls cannot withstand the blood flow(125). Furthermore, as most electrospun scaffolds are populated with cells and then cultured, it is also helpful to determine, if post culture there are any effects or changes to the mechanical properties of the scaffolds.

Mechanical properties are measured using a uniaxial tensile test (Figure 1.7) or computational analysis that avoids the need for destructive testing(126). Sample thickness is measured using a micrometer and then clamped with two

grips in a tensiometer at fixed distance between the grips. A small load cell is selected and a ramp test is then applied. The first failure point or plateau is recorded as the load at failure (or ultimate tensile strength, UTS) and the slope of this plot is calculated for the Young's modulus both shown as MPa (N/mm^2). The Young's modulus can be thought of as a measurement of the elasticity of the material. The tensile strength of scaffolds can be compared to native tissue that they are aiming to replace. Furthermore, the mechanical strength can be modified by the choice of polymer and any of the electrospinning conditions as outlined above to achieve a material that is suitable for a particular application. Studies on skin have shown that mechanical strength decreases with age and is constant in both men and women. This can be explained by the fact that the ratio of chondroitin-sulfate /keratin sulfate, the GAGs that absorb shear forces decrease in the dermis with age(127).
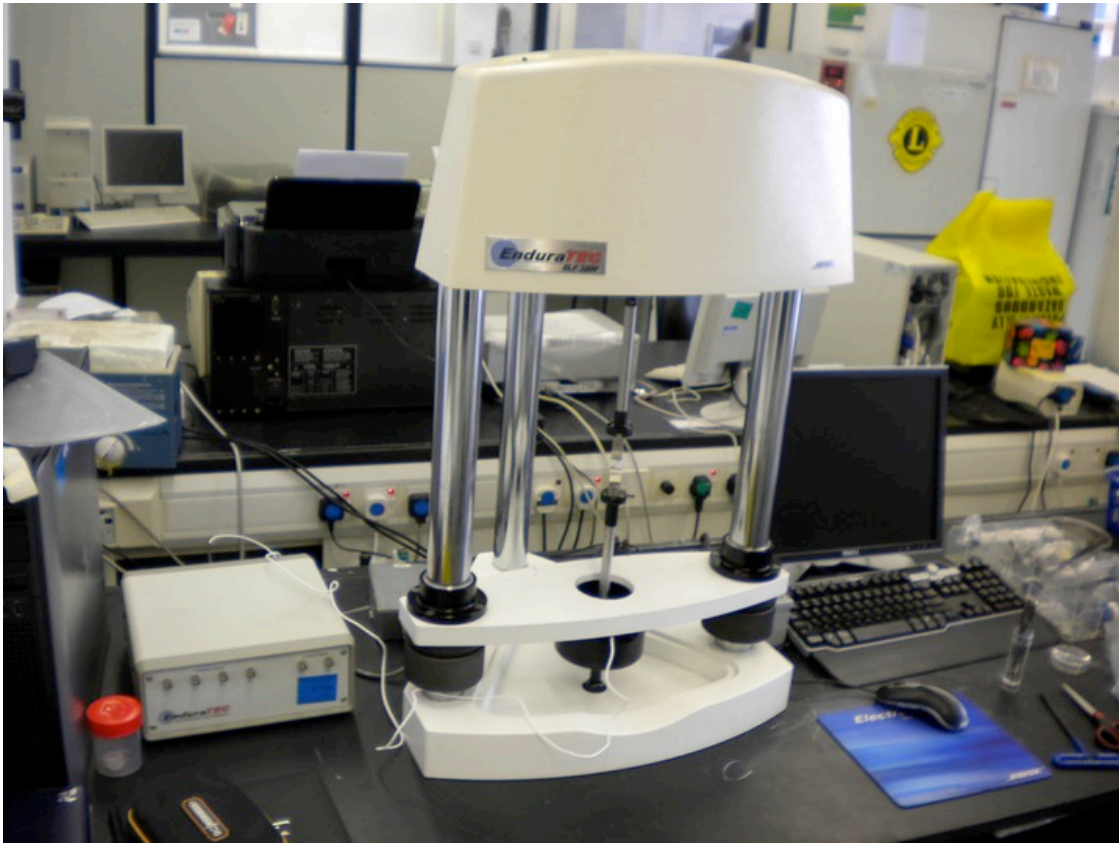
Figure 1.7: *Bose Tensiometer*

1.6.5 Modifications of electrospun scaffolds

Early work in electrospinning focused mainly on single polymers that resulted in well-aligned fibres. However, it is known that most tissues are not structured in such a predictable and organized fashion. Resident cells are spatially organized depending on the tissues concerned. For example, in skin, cells are stratified and its micro topography is complex and variable depending on the area of the body being studied. This lead to the use of layered micro and nano fibrous scaffolds

using different polymers to support specific cells that would better define the various layers of skin. This technique has been established by Bye et al using trilayers of polyhydroxybutyrate-co-valerate (PHBV) and PLA (114). Electrospinning of PHBV results in nanofibres that are useful as a barrier layer due to the small fibre and pore size between them. Scaffolds containing nanofibres facilitate cell adhesion, migration and proliferation, especially fibroblasts that assume a spindle-type shade in the neighbourhood of the nanofibres(128). Furthermore these ultra-fine fibres have similarities with the extra cellular matrix of natural tissue due to their porosity, fibre diameter orientation and mechanical strength(129).

Another technique employed to improve the topography of electrospun scaffolds involves the use of conductive templates onto which fibers are spun – this patterns the scaffolds thereby increasing the chances of cellular proliferation (Figure 1.8)(130),(131).

These templated scaffolds have already been developed for clinical application in the area of corneal transplantation. Traditionally limbal biopsies from the functional cornea are transplanted onto the contralateral diseased cornea by mincing it into very fine pieces. These are then laid onto a scaffold for support, which was initially amniotic membrane. Advances in tissue engineering resulted in the use of electrospun biosynthetic scaffolds which have been designed to

replace the amniotic membrane(132). Work by Ortega and MacNeil sought to further improve these scaffolds by mimicking the native morphology of limbal stem cell niches by templating the scaffolds using metal collectors (Figure 2.2)(133).

Within the body almost no cell is no more than 100-200 micrometres from the nearest capillary, within this spacing there is the ability for oxygen, nutrients and waste products to diffuse and maintain the viability of the tissues(134). Therefore, it is essential for any scaffold that is implanted in the body to become vascularised adequately at an early stage in order for it to survive and facilitate cellular migration and proliferation. The slow development of new blood vessels after in vitro implantation of 3D tissue engineered constructs especially if they are 1mm or thicker is a major challenge facing the sustainable translation to the clinical environment. Therefore, electrospun scaffolds themselves have been modified either physically or chemically to be able to better induce new blood vessel growth when implanted.

One such strategy involves functionalization of electrospun scaffolds. This is a process whereby known pro-angiogenic molecules are incorporated into the electrospinning process. For example heparin is a commonly used anticoagulant that can be linked to the surface of the scaffolds, this results in a surface that is

hydrophilic and negatively charged thus preventing absorption of molecules that promote thrombus formation (albumin and fibrinogen) in scaffolds to be used for vascular grafts(135). Moreover, it can act as a stabilizing carrier molecule for cytokines like vascular endothelial growth factor (VEGF). Heparin binds to the VEGF thereby allowing it to be delivered in a slow and predictable manner. VEGF on its own is believed to be a potent stimulator of angiogenesis and by coating the electrospun scaffold initially with heparin to bind to VEGF thereafter, it too can be functionalized onto scaffolds to increase potential neovascularization. This was demonstrated by Gigliobianco et al where electrospun PLA scaffolds coated with alternative layers of polyethylenelmine (PEI) and heparin showed that this was a superior technique to bind to VEGF as compared to using PEI and Polyacrylic acid (PAC) alone. Furthermore these heparin-coated scaffolds were then implanted into a chick chorionic allantoic membrane (CAM) assays which revealed better angiogenic activity in the heparin coated scaffolds(136).

Another important property of electrospun scaffolds surrounds their ability to support cellular function. It is known that ascorbic acid is a cofactor for the enzyme that crosslinks collagen fibrils that result in it functional triple helix structure(137). Ascorbic acid can be incorporated into electrospun scaffolds to

stimulate the production of collagen by fibroblasts. It has been demonstrated that by producing ascorbic acid releasing scaffolds, collagen production is increased significantly when they are seeded with fibroblasts compared to those scaffolds without ascorbic acid(110). The ability to stimulate collagen production is advantageous property of electrospun scaffolds in the fields of skin, cartilage, bone and soft tissue regeneration.
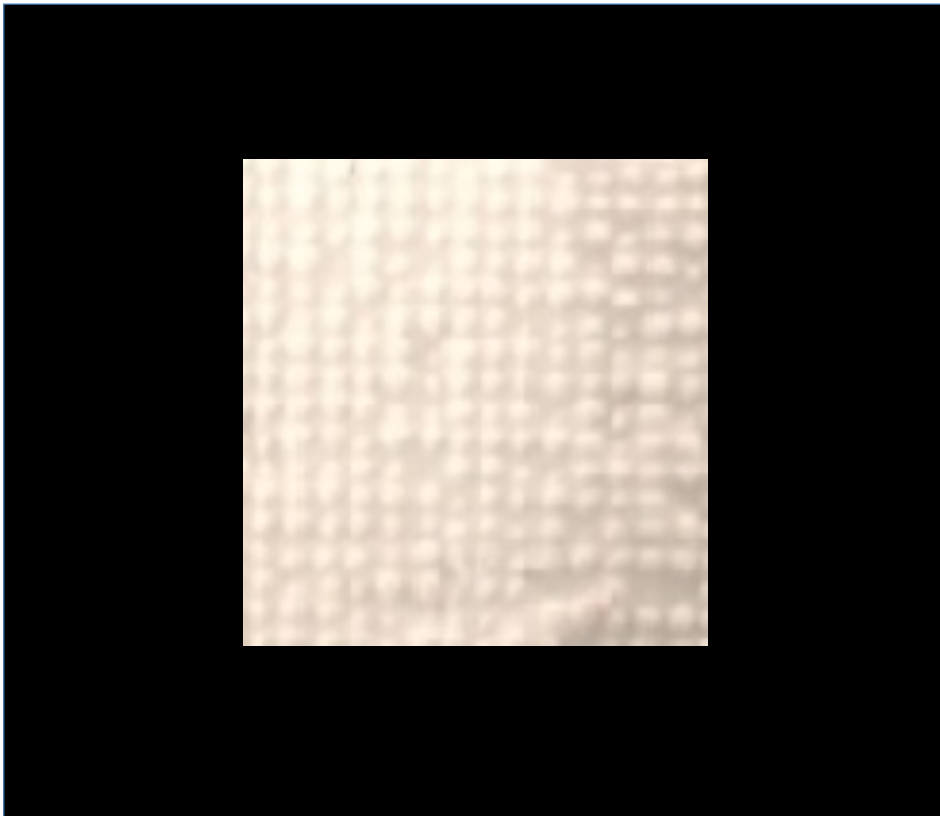
Figure 1.8: Templated trilayer electrospun scaffolds (x4 magnification)

1.6.6 Clinical Applications in Plastic and Reconstructive Surgery

There are many reasons why electrospun scaffolds serve as an attractive option for use in plastic and reconstructive surgery. Firstly they are safe, non-toxic and biocompatible. Scaffolds can be made to match the physical and mechanical profile of the tissue that it is aiming to regenerate. For example, the polymer chosen and electrospinning technique can be varied so that it provides a specific fibre size and pore dimension between the fibres to best suit the specific cell thereby tissue type that it aims to support. Furthermore, mixing polymers allowing it to provide support for, as long it is needed, can control its biodegradation. After this time period it is expected that the scaffold will degrade into physiological by products that are easily excreted leaving behind the patient's native tissue, which has regenerated, similar to an absorbable suture that disappears when the suture line has healed. Furthermore, scaffolds can be textured and moulded into different sizes and shapes to aid cellular attachment and proliferation.   Advancing technology has resulted in incorporation of various drugs and molecules that can allow scaffolds to be drug releasing and stimulate more rapid vascularization when implanted.

1.7 Tissue Engineering Approaches in developing a dermal analogue

Several research groups have attempted to produce tissue engineered dermal matrices using the electrospinning process. The scaffolds aim to match the tensile modulus that matches normal human skin and have a porous matrix to facilitate the proliferation of skin cells throughout its structure. Both non organic and biological polymers are used to create materials which are suitable for tissue engineered skin. One such group successfully used poly[lactic acid-*co*-glycolic acid] (PLAGA) which produced appropriate electrospun scaffolds for skin replacement(138) Other groups are trying to integrate bioactive components like glycosaminoglycans (GAGs) (hyaluronan and chondroitin sulphate) into scaffolds which has the effect of stimulating cellular proliferation and accelerated epidermal-dermal regeneration(139). Another challenge with tissue engineered scaffolds is the ability for cells to adhere to the scaffolds and thereby migrate and proliferate. As a result, techniques have developed which would serve to enhance this. For example PGLA (75/25) scaffolds when coated with collagen showed that keratinocyte proliferation increased significantly(140).

The first potential use of electrospun scaffolds is in the field of skin replacement. Full thickness wounds are by definition devoid of the dermal regenerative elements that facilitate healing. Many currently employed skin substitutes are

either dermal in origin, requiring a second procedure to obtain epithelial cover and are composed of products of animal origin, which although approved by the FDA still carries the risk of prion diseases. Additionally, many cultures are not accepting of materials that come from commonly used bovine and porcine sources. Electrospun scaffolds in this respect are desirable materials as they are biocompatible, synthetic and their chemical and physical structure can be altered to match key features that are required in skin reconstruction. These include prevention of fluid and protein loss from the wound bed, removal of exudate, inhibition of microbial invasion and their fibres need to support cellular attachment, guide endogenous cells to proliferate and remodel. Both natural and synthetic polymers have been used in the electrospinning process to produce electrospun scaffolds for skin regeneration. Improvements have been made to this technique by combining synthetic polymers that provide the mechanical strength of the scaffold and natural polymers like gelatin, chitosan and collagen to facilitate cellular attachment and migration. For example PCL/collagen scaffolds(141) and PCL/chitosan(142) have shown good fibroblast proliferation and adhesion which are potential applications in skin regeneration.

1.8 Fibrin

1.8.1 Introduction

Fibrin is the end result of the coagulation cascade which results in activation of thrombin. Thrombin in the presence of calcium ions cleaves fibrinogen to form fibrin. Fibrin are initially peptide monomers that are covalently cross linked though the action of factor 13 to form an insoluble polymerized fibrin clot(143). Fibrin is an essential component of the blood clot that has a central role in haemostasis. It has been used extensively as a biological polymer in tissue engineering. It is classed as a hydrogel and is structurally similar to the extracellular matrix that is present in tissues.

1.8.2 Clinical Applications of Fibrin

In clinical practice fibrin is used both as a sealant and haemostat. These functions although slightly different requires the fibrin to have a dense compact structure in order to plug the end of bleeding vessels and be strong enough to withstand shear mechanical forces that are applied to a wound. It is the most effect physiological tissue adhesive that is commercially available. The concentration of fibrinogen and thrombin used affects the structure of the clot and thereby influences its potential applications. Higher concentrations of

fibrinogen renders the clot mechanically stronger which is ideal if it is being used as an adhesive. Higher concentrations of thrombin results in a clot that sets very quickly which is useful in haemostasis. Fibrin itself is also a chemoattractant in wound healing cells to cells like fibroblasts and keratinocytes and their associated growth factors. Most commercial preparations of fibrin contain an anti-fibrinolytic component to slow the degradation of fibrin. Fibrin is used in various specialities depending on need. Its uptake has become popular for sealing dead spaces eg seroma pockets, bleeding wound beds when no source vessel is found, skin graft adhesive and even in solid organ lacerations eg liver. That being said, fibrin can be sourced from human and bovine sources and as a result there is always the potential risk of viral infection or foreign body response.

1.8.3 Use of Fibrin in tissue engineered bioproducts

Fibrin can have various applications in tissue engineering. Firstly, it can be used to support cells on its own i.e. cells can be suspended in the fibrin and cultured for transplantation. Additionally, it can be used with other polymers as part of a scaffold. This is advantageous as it utilizes the strengths of polymers for structure and mechanical strength and fibrin which incorporates as much cells as possible, thereby enabling good cell distribution which ultimately provides

coherent tissue development. Finally, fibrin can be used as a delivery for growth factors or other therapeutic agents. With regards to skin, fibrin has been used as a delivery vehicle for keratinocytes both in the form of gel sheets (144) and microbeads(145). It has also been used as a component part of various scaffolds. This is advantageous as fibrin has been shown to stimulate the migration of keratinocytes(146) and fibronectin which is a glycoprotein in fibrin does enhance cellular migration during wound healing(147).

For this thesis, the fibrin used in the experiments act as an adhesive to help retain the skin explants on the scaffolds and it is not integrated into the electrospinning process formally. Furthermore, it is not used as a cell delivery vehicle as we aim to use composite tissue pieces versus isolated cells to populate our electrospun scaffolds. This can be viewed as beneficial for many reasons. Firstly, it allows for fibrin to be used as part of the application rather than adding fibrin to the electrospinning process changes the mechanical structure of the electrospun scaffold which has already been established in the MacNeil laboratory for skin replacement. Moreover, by using fibrin on top of the scaffolds, its properties of both an adhesive and a chemoattractant to keratinocytes are used. By fixing the explants to the scaffolds thereby increasing the contact time of the explants to the scaffolds, facilitates cellular migration

from the explants onto the scaffolds which is essential if explants are to be used as a cell source.

1.9 Aims, Objectives and Rationale

1.9.1 Aims

The aim of this project is to design and evaluate a novel biodegradable dermal alternative (composed of medical grade PGLA (75:25) and PHBV) to be combined with skin explants for use as a one-step approach in theatre for reconstruction of full thickness skin defects.

The following are the hypotheses to be tested:

1. Culturing skin pieces on a trilayer scaffold consisting of micro/nano fibres will lead to better epithelial-stromal organization than would be achieved on a scaffold consisting of microfibers alone

2. Inclusion of features that approximate the dimensions of the rete ridges will help in the outgrowth of epithelial cells into the electrospun scaffolds.

3. The inclusion of fibrin as tissue glue will act as a stimulant for epithelial outgrowth into these synthetic scaffolds.

4. The presence of fibroblasts precultured in synthetic scaffolds help stimulate the outgrowth of epithelial cells from skin explants

1.9.2 Experimental Objectives

To achieve the above, the project was divided into the following experimental objectives:

1. Evaluation of how a novel method for spinning a basement membrane substitute can encourage epithelial-stromal organisation.

2. Investigation of whether the inclusion of features that approximate the dimensions of rete ridges will help in the outgrowth of epithelial cells into epithelial cells into the electrospun scaffolds.

3. Assessment of whether the inclusion of fibrin as tissue glue will act as a stimulant for epithelial outgrowth into these synthetic scaffolds.

4. Evaluation of whether inclusion of fibroblasts in the templated trilayer PGLA 75:25 / PHBV electrospun scaffolds will promote epithelial cell outgrowth in the presence and absence of fibrin.

1.9.3 Rationale

Traditional reconstructive plastic surgery techniques rob Peter to pay Paul. Skin grafting remains the gold standard for resurfacing full thickness burns and later functional reconstruction of scars and contractures. This poses problems in large burns (> 30% TBSA) where lack of donor sites results in multiple theatre sessions utilizing remaining unburnt skin sites and resulting in donor site morbidity.

The aim of this project is to bring the use of a one stage reconstructive technique using tissue-engineered scaffolds closer to clinical use. This promises to be a viable economical alternative, which uses the patient's own skin in the form of a biopsy, thereby avoiding the need to culture cells in the lab in combination with the use of biodegradable synthetic FDA approved polymers, already in clinical use, free from products of animal origin.

This model of combining scaffolds with small tissue explants is clinically successful in the treatment of scarred corneas. Ocular disease resulting in limbal stem deficiency results in the inability to use corneal transplants. A biopsy of

limbal tissue harvested from the contralateral healthy eye was minced into several pieces and spread onto amniotic membrane. Using fibrin glue to hold the pieces in place, this was grafted onto surgically prepared corneas in a single theatre session(132). At 6 week follow up stable epithelized corneas were seen. This study highlights that it is possible to combine tissue biopsies to regenerate new epithelium in situ. This is what we are aiming to achieve for skin epithelium regeneration.

We aim to have a similar model for skin. Clinically this would translate as follows: a small biopsy of the patient's skin is minced into very fine explants and combined with electrospun scaffolds and secured onto the surgically prepared wound bed. It is expected that fibroblasts and keratinocytes will migrate along the fibres of the scaffold to enhance wound healing without the need for culturing cells in a laboratory and further theatre sessions. The current project seeks to develop this further through the development of more sophisticated electrospun biodegradable membranes, containing 3D features to mimic the micro topography of the basement membrane and rete ridges.

Furthermore, we aim to assess the role of fibrin as a biological stimulator of the migration of skin cells from the explants onto the scaffolds. Fibrin is component of haemostasis and used surgically as sealant. It can be prepared from autologous plasma and is available as an FDA approved product for clinical

applications(148). The approach we describe could become a key addition to the plastic surgeon's armamentarium in treating full-thickness skin defects and in helping manage patients with burns that ideally require reconstruction using full thickness skin grafts but lack available donor sites.

Chapter 2

Materials and Methods

2.1 Ethical Permission for use of human skin

Ethical Permission was obtained from the NHS Health Research Authority, Yorkshire and Humber, Sheffield for the use of excess unwanted skin from reconstructive cases being undertaken in the plastic and reconstructive surgery department sited both at the Royal Hallamshire Hospital and Northern General Hospital under a research tissue bank license (number 15/YH/0017) (Appendix 2). This allowed us to obtain skin from any operative case that resulted in excess skin +/- subcutaneous fat that would normally be incinerated. Patients were consented preoperatively using both the standard Sheffield Teaching Hospitals Trust consent form in addition to that produced by Professor Sheila MacNeil, approved by the Sheffield Research and Ethics Committee (Appendix 1). An honorary clinical contract was obtained from the human resources department at the Sheffield Teaching Hospitals in order to facilitate this activity. As part of the consent process, it was a requirement to provide the patient with a written patient information leaflet, which contained a detailed explanation of why and how the skin was collected, its general use and storage. Furthermore, it provided essential contact details of the research team in case the patient had any queries at a later date (Appendix 1). Patient anonymity was maintained throughout in accordance with the General Medical Council's (GMC) Good Medical

Practice(149). The tissue was labelled and stored in an approved facility in keeping with the guidelines laid out by the Human Tissue Act (HTA) 2004(150).

2.1.1 Harvesting Donor Skin

A Watson knife was procured from York Medical Technologies Limited and disposable blades were obtained from Swann Morton Ltd, Sheffield, England. Skin was obtained in theatre from breast specimens (breast reductions or mastectomies) or brought into the lab for harvesting in cases of free flap breast reconstruction. The method used at either site was the same. Skin was harvested under aseptic conditions. One side, the specimen was secured onto a Styrofoam board with multiple 21G hypodermic needles, (Fisher Scientific, Loughborough, UK). The board was made aseptic by covering it with two layers of a sterile plastic covering. The other end of the tissue was manually stretched to tension the specimen appropriately. A sterile No. 22 blade (Swann Morton, Sheffield, UK) was mounted on a scalpel and placed into the space between the blade and the Watson knife handle to set the appropriate thickness (approximately 2mm). Once the skin was harvested it was placed into a 100mls universal sterile pot containing a mixture of phosphate buffered saline (PBS), penicillin/streptomycin (50 i.u/ml and 50µg/ml) and fungizone (313µg/l) (Sigma-

Aldrich, Dorset, UK). All specimens were then labelled with a unique number that was anonymous and not traceable to the patient. The skin collected was recorded in a human tissue log with the following details: date of collection, tissue collected and person collecting it. All authorized users thereafter were required to complete the user section in the log before taking the tissue for further use. Specimens were stored in a refrigerator at 4°C. After 3 weeks, any unused skin +/- subcutaneous fat was sent for incineration as per the Human Tissue Authority (HTA) requirements. Skin explants used from these specimens were prepared on the day of skin harvest and cell isolation was performed within four days.

2.2 Culture Media

2.2.1 Materials

The following reagents were made up in the lab for use in both 500mls 10% serum Green's media and 10% serum (Dulbecco's Modified Eagle's Medium, Sigma-Aldrich, Dorset, UK) DMEM respectively.

- Penicillin/streptomycin (Sigma-Aldrich, Dorset, UK) consisted of 10,000 i.u/ml penicillin and 10,000µg/ml Streptomycin. Final media concentration: 100 i.u/ml penicillin and 100 µg/ml streptomycin

- Fungizone (Sigma-Aldrich, Dorset, UK): Amphotericin B 250µg/ml, final media concentration: 0.625µg/l

- Adenine (Sigma-Aldrich, Dorset, UK): 2g of adenine powder was added to 280 ml of sterile distilled water and then acidified in 1M hydrochloric acid (HCL) until dissolved. This was made up to 320 ml using distilled water. It was then filtered to sterilize. Final media concentration: 184 µM

- Insulin (human recombinant) (Sigma-Aldrich, Dorset, UK) 1mg/ml: 250mg of human recombinant insulin was weighed out and to this 25mls of 0.01M HCL was added to dissolve the powder. 225 ml of distilled water was then added. It was then filtered to render it sterile. Final media concentration: 5µg/ml

- 3,3,5 –Tri-iodo-L-thyronine (T_3)/Apo-Transferrin (TT) (Sigma-Aldrich, Dorset, UK): 13.6mg T_3 was dissolved in 0.02M sodium hydroxide (NaOH). The volume was then made up to 100ml using distilled water and filtered to render it sterile. 100mg of apo-transferrin was dissolved in 12 mls of distilled water over 30-60 minutes. 0.2ml T_3 was then added and volume made up to 20ml using distilled water, this was again filtered to render it sterile. Final media concentration: T_3: 1.36 µg /l and transferrin 5mg/l

- Hydrocortisone (Sigma-Aldrich, Dorset, UK): 50mg of hydrocortisone was dissolved in 2ml sterile distilled water and to this 18mls of sterile PBS was

added. It was further sterilized using a filter. Final media concentration: 4 μg/ml

- Epidermal Growth Factor (Human recombinant) (EGF) (R&D systems, Biotechne): 20μl foetal calf serum was added to 2mls acetic acid (10mM). 1ml of this was added to a vial containing 200μg EGF powder and mixed to dissolve. 25μl aliquots were used in making media. Final media concentration: 10μg/l

- Cholera Toxin (Sigma-Aldrich, Dorset, UK): 1mg Cholera Toxin was dissolved in 1.18ml distilled water. 0.1ml of this solution was added to 10ml medium with serum to form 8.47pg/l stock. Final media concentration: 8.47 pg/l.

2.2.2 10% Serum Green's Media

This was used for keratinocyte culture and used in all experiments. It was made under sterile conditions by combining the following components at room temperature in a Class II laminar flow hood: 108mls Nutrient Mixture F-12 HAM, (Sigma-Aldrich, Dorset, UK) 330 mls of DMEM, 5mls of penicillin/streptomycin, 1.25mls of fungizone, 50 mls of foetal calf serum (Sigma-Aldrich, Dorset, UK), 2mls adenine, 2.5 mls insulin, 0.5mls TT, 0.08ml hydrocortisone, 0.025ml EGF

and 0.5mls of cholera toxin. This was labelled, dated and stored in a sterile 500ml Duran bottle at 4°C in between uses.

2.2.3 10% serum DMEM

This was used for fibroblast culture and was made and stored in the same conditions as above by combining the following reagents: 440 mls of DMEM, 5mls of penicillin/streptomycin, 1.25mls of fungizone, 50 mls of foetal calf serum, 2mls adenine, 2.5 mls insulin, 0.5mls TT, 0.08ml hydrocortisone, 0.025ml EGF and 0.5mls of cholera toxin.

2.2.4 Serum free Media

10% serum free Green's media and 10% serum free DMEM was used when labelling fibroblasts and keratinocytes with the fluorescent markers CellTracker green (CMFDA) and CellTracker red (CMTPX) (Invitrogen, USA) respectively. The conditions for producing and storing the media were the same as above. The reagents for 10% serum free Green's media were: 120mls Nutrient Mixture F-12 (HAM), 367 mls of DMEM, 5mls of penicillin/streptomycin, 1.25mls of fungizone,

2mls adenine, 2.5 mls bovine insulin, 0.5mls TT, 0.08ml hydrocortisone, 0.05ml EGF and 0.5mls of cholera toxin. For 10% serum free DMEM, the following reagents were combined: 489 mls of Dulbecco's Modified Eagles' Medium, 5mls of penicillin/streptomycin and 1.25mls of fungizone.

2.3 Cell Culture

2.3.1 Materials

The following reagents were prepared in the laboratory for use in cell culture:

- Difco-Trypsin 0.1% (w/v) was prepared by adding 0.5g of Difco trypsin powder (Difco, West Molesey, Surrey, UK) to 0.5 g D-glucose and 500-µl phenol red and topped up to 500mls PBS. The pH was made to 7.45 using 2M NaOH and sterilized by filtering. It was stored and used in 10 mls aliquots for cell culture purposes.

- Collagenase A from the bacterium Clostridium histolyticum was made by dissolving 100mg collagenase A powder (Roche Laboratories, West Sussex, U.K) in 180mls 10% serum free DMEM. This was then filtered to sterilize and 20mls of foetal calf serum (FCS) was then added. This

resulted in a 0.05% (w/v) solution. The solution was used in 10ml aliquots for fibroblast isolation.

2.3.2 Preparation of irradiated mouse 3T3 (i3T3) fibroblast feeder layers on T75 flasks

Green's media containing 10% serum was warmed and a Class II tissue culture cabinet was prepared. Using the appropriate safety gear, the required number of cryovials containing i3T3 cells (irradiated with a dose of 60 Greys) were removed from the liquid nitrogen dewar and placed in a 37°C incubator until just thawing. The thawed cells were transferred to a sterile 30 ml universal tube containing 10% serum Green's media. This was then centrifuged at 1000 rpm for five minutes. The supernatant was discarded and isolated cell pellet was resuspended in 5mls of 10% serum Green's Media. The cells were counted using the method outlined below. If needed more media was added to the cells to obtain a concentration of 1×10^6 cells/ml. An appropriate volume of cell suspension was then transferred to T75 flasks to obtain a final seeding concentration of 1×10^6 cells. Media was then topped up to 10 mls and this was incubated at 37°C, humidified atmosphere 5% CO_2 overnight. The cells were inspected the following day to observe for confluence and to exclude infection.

2.3.3 Keratinocyte Isolation from human skin

On the day of skin harvest, a sterile forceps was used to place the split thickness skin sample in a 90mm petri dish containing a small volume of PBS. A sterile No. 22 blade was used to cut the skin in 1cm^2 square pieces. The skin pieces were then transferred to a sterile 30 ml universal container containing 15mls of Difco –trypsin and incubated overnight at 4°C. After 12-20 hours, the skin suspension was removed from the refrigerator and a sterile forceps was used to remove one piece of skin from the Difco-trypsin, rinsed in sterile PBS then placed in a dry petri dish with the dermis side down. A few drops of PBS were added to keep the skin moist. The sterile forceps was then used to peel the epidermis away from the dermis. If this occurred easily, the Difco-trypsin was poured away into a waste pot without disturbing the skin. 10-15 mls of FCS was added to the bottle to negate the action of the Difco- trypsin. If the epidermis did not peel away easily from the dermis then the skin suspension containing the Difco-Trypsin was returned to 4°C until the epidermis was easily removed.

The process of peeling away the epidermis was repeated for all the skin squares. A sterile No. 22 blade was then used to very gently scrape the cells from both the papillary surface of the dermis and the underside of the epidermis. The epidermis was then placed in a labelled petri dish for incineration and the dermis

was kept aside for later use in fibroblast culture. As the cells are scraped from these surfaces the PBS became increasingly cloudy. A sterile Pasteur pipette was used to collect the PBS containing the cells, which was then added to a sterile 30 mls universal tube containing 10-15ml of 10% serum Green's media.

2.3.4 Keratinocyte Culture

Once the keratinocytes were harvested from all the skin pieces, the 30 ml Universal tube was centrifuged at 1000 rpm for 5 minutes. The supernatant was decanted and the cell pellet was broken by tapping the tube on the base of the culture cabinet. The cells were re-suspended in 10 mls of 10% serum Green's media and mixed thoroughly. The cells were then counted (see cell count and viability assessment below). Depending on the cell count, the volume of 10% serum Green's media was adjusted to give a final concentration of 1×10^{6} cells/ml. A sterile plastic pipette was then used to dispense an appropriate volume of cell suspension into T75 flasks containing a feeder layer of i3T3 cells (see section on preparing a feeder layer of i3T3 cells). As a guide, primary (p1) keratinocytes were seeded not less than 1.5×10^{6} cells per flask. The volume was made up to 10-13 mls per flask by adding 10% serum Green's as appropriate. The cells were spread gently across the flasks by gently sliding the flasks from

side to side and then placed in an incubator at 37°C, humidified atmosphere 5% CO_2 overnight. After 48 hours in culture, the flasks were examined under a light microscope for confluence and to exclude infection. The media was changed as appropriate. This continued until cells were 60-70% confluent. At this point they were either passaged, subjected to freezing or used in experiments.

2.3.5 Passage of Keratinocytes

This occurred when the keratinocytes were about 60-70% confluent. 24 hours prior to passaging the keratinocytes for expansion, feeder layers of i3T3 cells in T75 flasks were prepared. 10% serum Green's media, 0.02% EDTA solution (Sigma-Aldrich, Dorset, UK) and 5mls of trypsin-EDTA (Sigma-Aldrich, Dorset, UK) were warmed to 37°C in the incubator before use. A sterile plastic pipette was used to remove all the culture medium from the T75 flask. The flasks were then washed three times with PBS. 5mls of 0.02% EDTA solution was added to each flask and returned to the 37°C incubator for five minutes. After this time the flasks were removed from the incubator, the sides were gently tapped and they were viewed under the light microscope to assess for evidence of detached i3T3 cells. If necessary, the flasks were returned to the 37°C incubator for a further five minutes. Once the i3T3s were detached the EDTA was removed and discarded. 2mls of trypsin- EDTA was then added to the flask, gently swirled and

incubated at 37°C for 10 minutes. After this time, they were removed from the incubator, the sides gently tapped and viewed under the light microscope to ensure that all keratinocytes were detached. Once confirmed, the trypsin- EDTA cell suspension was placed into a sterile 30 ml universal tube. To this an equal volume of 10 % serum Green's was added. It was then centrifuged at 200 g (1000 rpm) for 5 minutes. The supernatant was decanted and the pellet was dislodged by tapping the tube on the base of the culture cabinet. The cells were then re-suspended in 5 mls of 10 % Green's medium and mixed thoroughly and then counted. If needed more 10% serum Green's medium was added to give a final concentration of 1×10^6 cells per ml. A sterile pipette was then used to dispense an appropriate volume of the cell suspension into a T75 flask containing a feeder layer of i3T3 cells. As a guide, p2 and p3 keratinocytes were seeded at 2×10^6 cells per T75 flask. The flasks were topped up with an appropriate volume of 10% serum Green's media and placed in an incubator at 37°C, humidified atmosphere 5% CO_2 overnight. The cells were inspected daily under the microscope and the media changed every 2 -3 days thereafter. At 60 – 70 % confluence the cells were passaged, frozen or used for seeding. For these experiments p1-p3 keratinocytes were used.

2.3.6 Fibroblast Isolation from Human Epidermis

Using the small squares of split thickness dermis that was denuded of epidermis from keratinocyte isolation, a sterilized No. 22 blade was used to mince it into very fine pieces (2-3mm^2). A sterilized forceps was used to transfer the pieces of dermis into a sterile petri-dish containing 10mls of Collagenase A. This was incubated in 37°C, humidified 5% CO_2 overnight. The following day, the Collagenase A/dermis suspension was inspected to ensure that all the dermis was digested (cloudy appearance). Any pieces of residual dermis were removed using a sterile forceps and discarded into a labelled 30ml universal tube for incineration.

2.3.7 Fibroblast Culture

The fibroblast suspension was decanted into a sterile 30 ml universal tube, and centrifuged at 400g (2000 rpm) for ten minutes. The supernatant was discarded carefully and the remaining cell pellet was dislodged by tapping the tube on the base of the culture cabinet. The cells were then resuspended in 5 mls of serum 10% DMEM. They were then counted (see counting cells) and if needed further 10% serum DMEM was added to obtain a concentration of 1 x 10^6 cells per ml. The cell suspension was then transferred into T25 flasks (25 cm^2), as a guide 0.5-

1×10^6 primary (p1) fibroblasts were seeded per flask. The media volume in each flask was made up to 7 ml. The cells were spread across the flasks by gently sliding the flasks and incubated at 37°C, in a humidified atmosphere of 5% CO_2. The cells were inspected daily under the light microscope for confluence and to exclude infection. The first media changed occurred at 48 hours and three times a week thereafter. This was continued until the cells became 60-70 % confluent (usually 5-7 days).

2.3.8 Passage of fibroblasts

This was performed when the cells were 60-70 % confluent. The flasks of fibroblasts were removed from the incubator, media discarded and washed three times with sterile PBS. 3mls of Trypsin-EDTA was added to each flask and incubated at 37°C, in a humidified atmosphere of 5% CO_2 for five to ten minutes. They were then removed from the incubator and the sides of the flasks were gently tapped to aid cellular dislodging. The flasks were inspected under light magnification to confirm that all the cells had detached. Once this was confirmed the fibroblast- trypsin EDTA suspension was placed into a sterile 30

ml universal tube. To this an equal volume of 10% serum DMEM was added. It was then centrifuged for 400g (2000 rpm) for 10 minutes. The supernatant was discarded and the cell pellet dislodged by tapping the base of the tube against the tissue culture cabinet. The cells were resuspended in 5 mls of 10% serum DMEM, mixed thoroughly and counted. If needed further 10% serum DMEM was added to obtain a final concentration of 1×10^6 cells per ml. The cellular suspension was then placed into newly labelled T75 flasks and topped up to 10-12 mls of 10% serum DMEM. As a guide p2 and p3 fibroblasts were seeded at 3×10^5 cells per T75 flask. The cells were then spread across the flasks and incubated at 37°C, humidified 5% CO_2 overnight. Cells were inspected daily to observe for confluence and to exclude infection with media changes every 2-3 days.

2.3.9 Cell count and viability assessment.

This was performed using Trypan blue (mixture of 0.4% trypan blue and PBS, Sigma-Aldrich, Dorset, UK). Once the cell suspension was centrifuged and the supernatant discarded the resultant cell pellet was mechanically dislodged by tapping it against the side of the tissue culture hood. To this a known volume of either 10% serum DMEM (for fibroblasts) or 10% serum Green's (for

keratinocytes) was added and mixed thoroughly. A 200µl micropipette and sterile tip was used to remove 50 µl of the cell suspension and added to 50µl of trypan blue in a sterile 7ml bijou. This was then mixed thoroughly by pipetting up and down 2-3 times. 50µl of this trypan blue- cell suspension was then added into the two counting chambers of a Neubauer haemocytometer (Fisher Scientific, Loughborough UK) and placed under a light microscope. The viable cells (unstained) were counted in each chamber and averaged for the two. This was then multiplied by the volume in mls of the resuspended cells and multiplied by 10^5 to obtain the number of cells in that volume of cell suspension.

2.3.10 Cell cryopreservation

Flasks containing cells were removed from the incubator, media discarded and washed twice with PBS. 5mls of trypsin/EDTA was then added to each flask and incubated at 37°C, in a humidified atmosphere of 5% CO_2 for ten to fifteen minutes. After this time, they were gently tapped against the sides of the culture cabinet to aid cellular detachment and inspected under the light microscope to confirm this. The cell/trypsin EDTA suspension was transferred to a sterile 30 ml universal tube and the trypsin deactivated by adding an equivalent amount of either 10% serum DMEM (fibroblasts) or 10% serum Green's (keratinocytes). It was then centrifuged at 200 g (1000 rpm) for 5 minutes. The supernatant was

discarded and cells resuspended in 5mls of media. The cells were then counted.

Freezing media was made by adding 3mls of dimethyl-sulphoxide (DMSO, Alfa

Aesar, USA) to 30 mls of FCS to make a mixture of 10% DMSO in FCS. Once

counted the cells were again centrifuged at 200g (1000 rpm) for 5 minutes. The

supernatant was discarded and the cellular pellet was resuspended in an

appropriate volume of the freezing medium to obtain a cell count of 1x 10^6 cells

per ml. A sterile pipette was used to add 1 ml of this cell/freezing medium to

labelled cryovials (CryoPure Tubes, Sarstedt AG &Co.) These were then placed

in a Mr Frosty (Sigma-Aldrich, Dorset UK) freezing container and into the -80°C

freezer for 2-4 hours. Following this the cryovials were transferred into liquid

nitrogen (-196°C) for long-term storage.

2.3.11 Thawing of cells

The required numbers of vials containing cells were removed from the liquid

nitrogen left to defrost at room temperature. They were then transferred to a

sterile 30 ml universal tube containing 10 mls of the appropriate pre-warmed

media depending on the cell type. The mixture was the centrifuged at 200g

(1000 rpm) for five minutes. The supernatant was discarded and the cell pellet

was resuspended in 5 mls of the appropriate media and counted using trypan

blue. Cells were then re-centrifuged or more media added to obtain a concentration depending on the need.

2.4 Preparation of Skin Explants

On the day skin harvest, 5 x 5 mm^2 square pieces of skin were cut using a sterile No. 22 Scalpel in a sterile petri dish in a few drops of PBS to keep the skin moist. These were then finely minced into approx. 16-20 squares. To this, a small amount of 10% serum Green's media was added to create a suspension. For each experimental construct mince from one square was used.

2.5 Preparation of Euro Skin

This was obtained from the Euro Skin Bank, Zeestraat, Netherlands. This is a national tissue bank that collects and preserves split thickness human skin in glycerol. Clinically it serves as a temporary wound dressing for wounds.

To prepare the Euro Skin, they were firstly rehydrated in a sterile 100ml universal pot using sterile PBS for 24 hours. Following this the PBS was discarded and the rehydrated skin was immersed in an excess 1M sodium chloride (NaCl) solution and incubated overnight at 37°C, in a humidified atmosphere of 5% CO_2. At this point the epidermis was visibly separated from the dermis. This made it easier to remove any remaining adherent epidermis with a sterile No. 22 blade.

The de-epithelized dermis (DED) was then washed twice with PBS. At this point, the DED was incubated with Green's media to remove any remaining sodium chloride for at least 48 hours before use. For experiments 15 x 15 mm^2 square pieces of skin was used in constructs.

2.5.1 Mechanical testing of Euro Skin and human skin used for explants

Mechanical testing was carried out in the MacNeil laboratory using the same method for electrospun scaffolds. Prior to mechanical testing, the Euro Skin was left to warm to room temperature. A sterile No. 22 blade was used to remove the epidermis and cut into 15 x 14mm rectangular strips. These were placed again in sterile PBS to ensure that they were hydrated. Thickness was then measured using a digital micrometer. Uniaxial tensile testing was performed in air at room temperature set at a rate of 0.1 mm/sec with a maximum displacement of 10 mm in the direction of the long axis of the samples. Analysis followed the method outlined below for electrospun scaffolds.

2.6 Preparation of Electrospun Scaffolds

2.6.1 Polymer Preparation

For these experiments electrospun scaffolds were made from two biodegradable synthetic polymers. The first one was Polyhydroxybutyrate/Polyhydroxyvalerate (PHBV) 12% biopolymer (Goodfellow, Cambridge limited, Huntingdon, England) which was used as the middle layer of the trilayer scaffold. Poly (D, L-lactide-co-glycolide) lactide: glycolide 75:25 (PGLA) (Sigma-Aldrich, Dorset, UK) was used on either side of the PHBV to complete the trilayer construct. PHBV was weighed out into a glass bottle. To this an appropriate quantity of 10 % wt./volume methanol and 80% wt. dichloromethane (solvent ratio 88.8:11.1 DCM-MeOH (Sigma-Aldrich, Dorset UK) was added using a glass pipette to obtain 10 % $^{wt.}/_v$ PHBV. The mixture was placed onto a stirrer and kept overnight for dissolving. 10 % $^{wt.}/_v$ PGLA (75:25) polymer solutions were similarly made by dissolving the polymer in the appropriate volume of dichloromethane (DCM) (Sigma-Aldrich, Dorset, UK) depending on the volume of the polymer that was required.

2.6.2 Electrospinning Conditions

The electrospun scaffolds used in these experiments were trilayer consisting of a middle core of PHBV and on either side PGLA (75: 25).

They were also templated with niches to mimic the rete ridges or the stem cell niches found in the dermal-epidermal junction of skin. These niches were templated onto the scaffolds using a textured metal collector (Figure 2.1).

The first polymer, PHBV was loaded into 4 x 5ml syringes (1 ml in each syringe) and fitted with a blunt tipped needle, 0.8cm internal diameter (Intertronics, Kidlington, UK). All four syringes were placed into a single syringe driver (Genie Plus, Kent Scientific, Connecticut, USA) with a fitted plastic backing to facilitate driving all four syringes at the same time.

The copper board collector was wrapped in one layer of aluminium foil with the shiny side facing the syringe driver. The metal templates were attached to the middle of the copper board using carbon conductive tape (Agar Scientific, Stanstead, UK) (Figure 2.2).

Adhesive tape was used to attach the prepared collector to a mandrel 20 cm wide and 10 cm in diameter. The spinning occurred under static conditions.

A perforated 6x6 cm copper plate was fitted over all 4 needles and a crocodile tip from a metal generator was attached to one of the needles. The needles were positioned 11cm from the copper collecting board when PHBV was used. The metal collector was then connected to a voltage generator set at 17kV and earthed. The syringe driver was turned on at a rate of 4mls/hour, ensuring that the diameter was set to 12.06mm. The voltage generator was then switched on

(Figure 2.3). The Taylor cones were removed with a wooden collector. Once the syringes of PHBV were finished, four new syringes containing the PGLA (75:25) were then loaded onto the syringe driver and fitted with the same blunt needle tips. The tip of the needle was now positioned 17 cm from the foil wrapped copper collector. The syringe driver had the same settings as the PHBV. The syringe driver was then turned on followed by the generator set at 17kV. Once the syringes containing PGLA (75:25) were empty, the scaffold was removed from the collector, turned over and re-attached to the copper board using adhesive tape. A further 4 x 5 ml syringes containing PGLA 75:25 were loaded onto the syringe driver and electrospun to form the final layer of the trilayer scaffold. Once completed, the scaffolds were stored in a sterile plastic bag and placed in a freezer at -12°C until use. In order to make non-templated scaffolds, the conditions were the same apart from omitting the use of the textured metal templates.
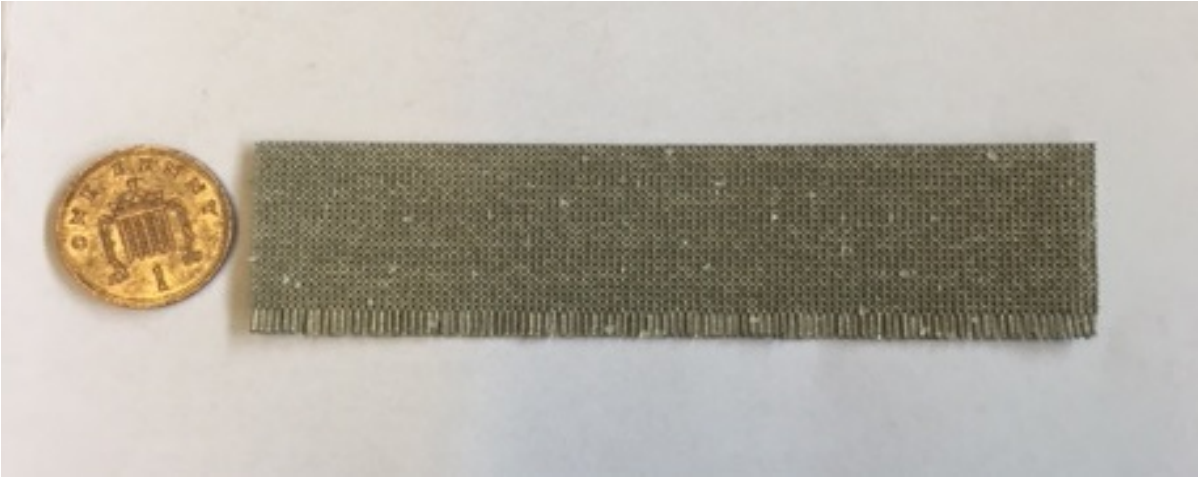
Figure 2.1: *Top: Metal template with coin next to it for scaling purpose, Bottom: illustration of the pattern of the metal templates to illustrate the small square structures used to imprint the template to aid creation of the niches.*
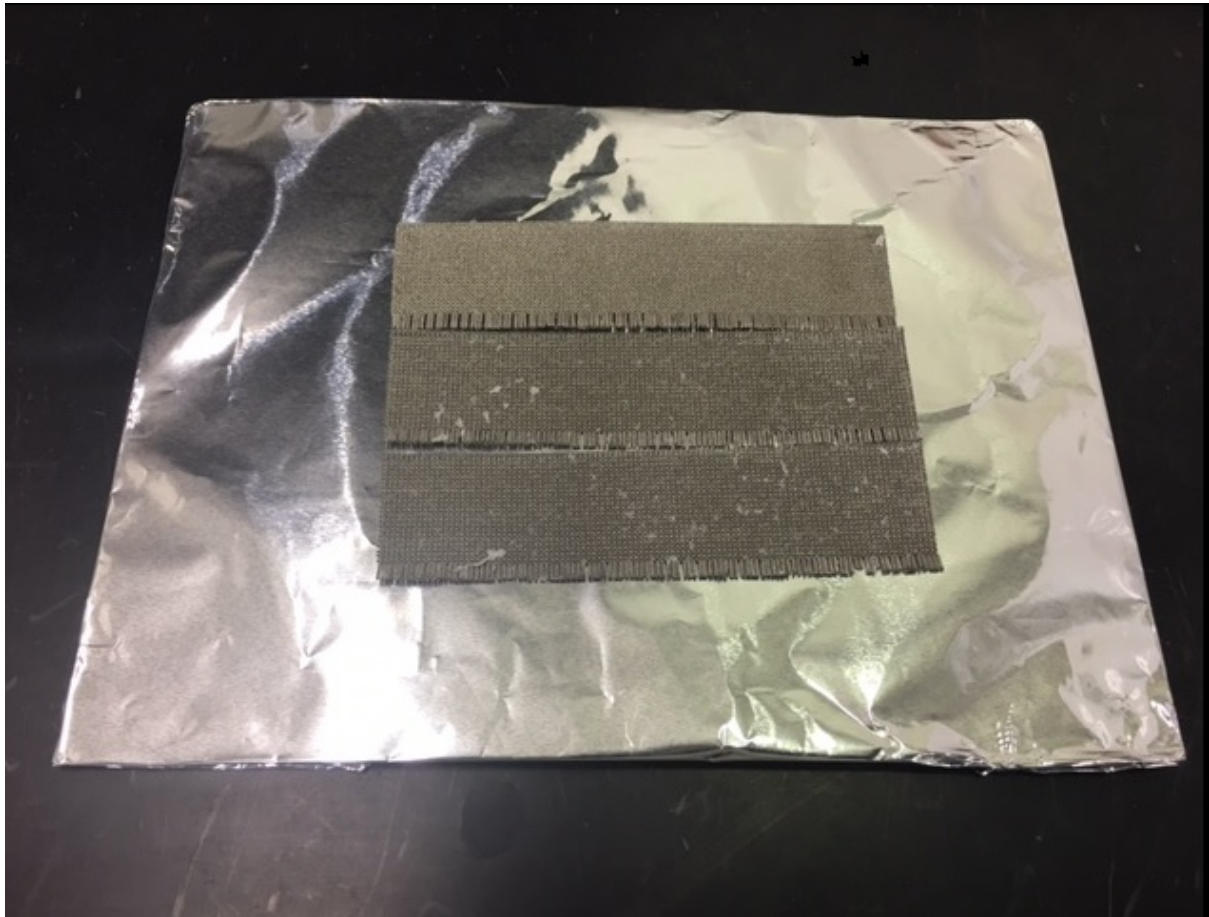
Figure 2.2 Conductive copper board wrapped in foil with the metal collectors attached using carbon conductive tape
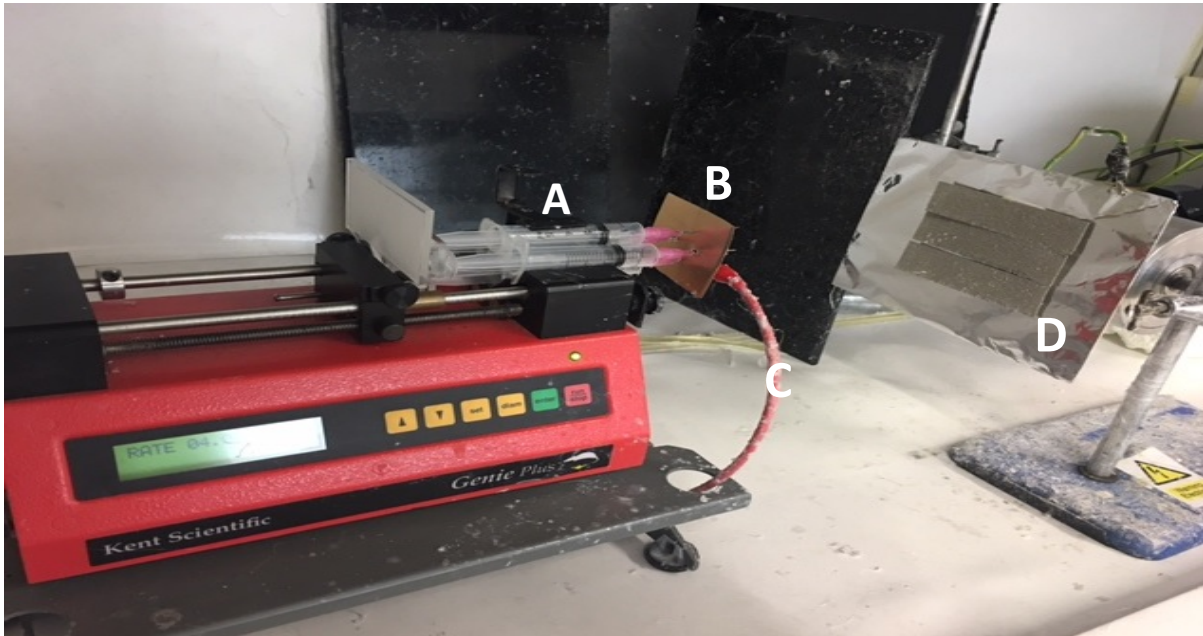
Figure 2.3: *Electrospinning process demonstrating the set up.*
A: loaded syringes with polymer, B: Copper Plate fitted to the Blunt tipped needles, C: Crocodile Clip connected to the 17kV Generator, D: Collector with Metal templates attached

2.6.3 Selective Laser Melt Collectors

These were obtained from Dr Ilida Ortega's research group based in the School of Clinical Dentistry at the University of Sheffield. These were manufactured using a Renishaw SLM 125 machine (Renishaw, Gloucestershire, UK) and 316L stainless steel. The plates were made using a range of niche like morphologies designed using the computer aided software Solidworks,

(Dassault Systèmes SOLIDWORKS Corp). The specific processing parameters for the fabrication of the collectors were as follows: laser power 200W; speed: 480nm/s; point distance: 50 μm and exposure time 70μs.

2.6.4 Sterilization of electrospun scaffolds

All scaffolds were sterilized before use, by methods previously established in the MacNeil Laboratory. Once the squares of scaffolds were cut to the desired size (usually 1.5x 1.5 cm^2 pieces) for use in experiments, they were placed in a sterile 100ml universal pot containing 70% ethanol (Fisher Scientific, Loughborough UK) for 15 minutes. Following this they were washed three times with sterile PBS and left to air dry in a tissue culture hood overnight. They were then used in experiments or stored in sterile plastic bags for later use in a freezer at -12°C.

2.6.5 Mechanical Testing of Electrospun Scaffolds

Mechanical properties were measured using a uniaxial tensile test. The device used in the laboratory to conduct this test was a uniaxial tensiometer (Bose Electroforce test instrument, Bose, Minnesota, USA). The calibration of the selected 22N load cell was checked before using the instrument and was found

to be satisfactory using linear regression analysis (R^2 = 0.9997). Samples were cut into rectangular pieces of at least 15mm by 4 mm, and measurements of width and thickness were obtained using a digital micrometer. Samples were clamped with two grips in the tensiometer, ensuring 10 mm in distance between the grips (Figure 2.4), and the device was pre-tuned to the particular scaffold that was being tested. A ramp test was applied at a rate of 0.1 mm/sec with a maximum displacement of 10 mm on the direction of the long axis of the samples. Stress was normalized by the area (width x thickness) and the strain by the tested length of the samples (all 10 mm). The values were analysed using an OriginPro 8 Software (obtained from the University of Sheffield). The first failure point or plateau was recorded as the load at failure (or ultimate tensile strength, UTS) and the slope of this plot was later calculated for the Young's modulus (YM) both shown as MPa (N/mm2). The displacement at the failure point was recorded as the Strain at the UTS (% of displacement from its original size).
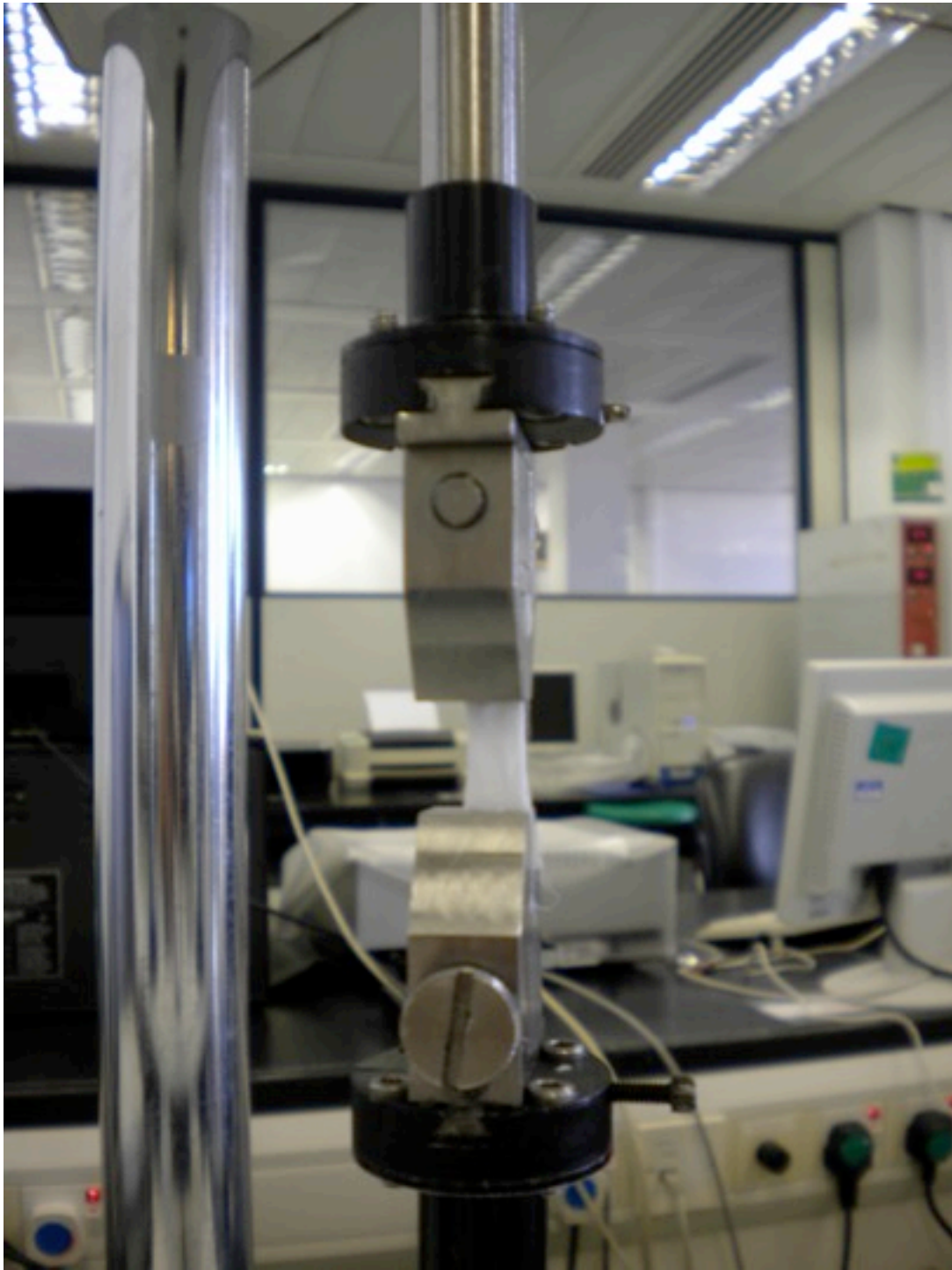
Figure 2.4 *Clamp Stretching Electrospun Scaffold during Mechanical Testing*

2.7 Fibrin Preparation

2.7.1 Materials

Fibrin was made by combining human fibrinogen with human thrombin.

18.75mg of Fibrinogen (human plasma; 50-70% protein, Sigma-Aldrich, Dorset, UK) was weighed out and added to 1ml of 0.9% NaCl (w/v).

To make 0.9% Normal Saline, 0.9g of NaCl salt was weighed out and added to 100mls of distilled water. The fibrinogen/ Saline mixture was placed into a water bath at 37°C for 4 hours to aid dissolving. This was then sterilized by filtering.

1 KU of Thrombin from human plasma (Sigma-Aldrich, Dorset) was dissolved in 4mls of distilled water to obtain a concentration of 250 IU/ml. This was then filtered to sterilize. These were then aliquoted into 100 µl portions for later use.

2.7.2 Methods

In order to create fibrin during experiments, the constituent components were defrosted in a water bath slowly over 2 hours until they were at room temperature. Depending on the concentration of fibrinogen and thrombin being used, the desired quantities were pipetted out- fibrinogen first followed by thrombin and mixed on the scaffolds. The explants were added and manipulated to allow for an even spread before the fibrin could set.

2.8 Experiments

2.8.1 Preparation of tissue engineered skin composites Using DED.

These constructs were used as controls. Medical grade stainless steel rings (1cm external diameter, $0.79cm^2$ area) and grids (Figure 2.5) were obtained from the Department of Medical Physics, Royal Hallamshire Hospital, Sheffield. A sterile No. 22 scalpel was used to cut the prepared Euro Skin into 15 x15 mm^2 pieces. The epidermis was gently scraped off, leaving the papillary dermis exposed. These were placed into 6 well plates with the reticular dermal side uppermost and the stainless steel metal ring was placed on top of it. To this, $1x10^5$ CMFDA fluorescently labelled fibroblasts (between passage 4 to 9) were placed in the centre of the metal ring suspended in 0.5mls of 10% serum DMEM. 2 mls of 10% serum DMEM was used to top up the surrounding Euro Skin outside the steel ring and these were incubated at 37°C, humidified 5% CO_2 for 48 hours. The constructs were then removed from the incubator and the presence of fibroblasts were verified by observing for CMFDA using an Olympus IX73 Epifluroscent Microscope. The media was then discarded and the scaffolds were then turned over with the papillary side uppermost. New sterilized metal rings were then placed onto the centre of the square pieces of skin and fluorescently labelled keratinocytes CMTPX were now seeded into the centre of the metal

rings. For this 3x 10^5 keratinocytes were used between passage one to four suspended in 0.5mls of 10% serum Green's media. 2mls of Green's media was then added outside the ring and this construct was again incubated at 37°C, humidified 5% CO_2 for 48 hours. At the end of this time, the 6-well plates were removed from the incubator and the presence of both keratinocytes and fibroblasts were assessed observing for CMFDA and CMTPX. Once cells were confirmed to be present, the metal rings were removed and the DED seeded with fibroblasts and keratinocytes were raised to an air liquid interface using the metal grids. These were then cultured for 7, 14 and 21 days respectively with media changes occurring three times a week.
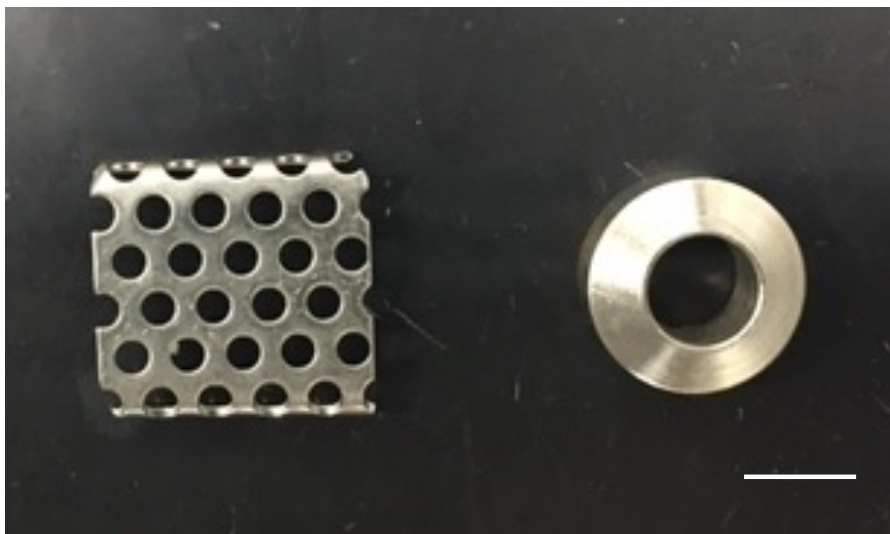
Figure 2.5: Metal rings and grids used in constructs obtained from the medical workshop of the Royal Hallamshire Hospital, Sheffield. Scale bar= 1cm.

2.8.2 Preparation of isolated keratinocytes and fibroblasts seeded on trilayer electrospun scaffolds.

A sterile No. 22 scalpel was used to cut sterilized trilayer electrospun scaffolds into 15 x15 mm^2 pieces. These were placed into 6 well plates followed by stainless steel metal rings placed on top. To this, 1x10^5 CMFDA fluorescently labelled fibroblasts (between passage 4 to 9) were placed in the centre of the metal ring suspended in 0.5mls of 10% serum DMEM. 2 mls of DMEM was used to top up the surrounding scaffold outside the steel ring and these were incubated at 37°C, humidified 5% CO$_2$ for 48 hours. The constructs were then removed from the incubator and the presence of fibroblasts were verified by observing for CMFDA. The media was discarded and the electrospun scaffolds were turned over. New sterilized stainless steel metal rings were then placed onto the centre of the square pieces of fibroblast seeded electrospun scaffolds and fluorescently labelled CMTPX keratinocytes were now seeded into the centre of the metal rings. For this 3x 10^5 keratinocytes were used between passage one to four suspended in 0.5mls of 10% serum Green's media. 2mls of 10% serum Green's media was then added outside the ring and this construct was again incubated at 37°C, humidified 5% CO$_2$ for 48 hours. At the end of this time period, the 6-well plates were removed from the incubator and the

presence of both keratinocytes and fibroblasts were confirmed by observing for CellTracker green and CellTracker red. Once cells were found to be present, the metal rings were removed and electrospun scaffolds containing fibroblasts and keratinocytes were raised to an air liquid interface, with the side containing the seeded keratinocytes uppermost using the metal grids for support. These were then cultured for 7, 14 and 21 days respectively. Media changes using 10% serum Green's occurred three times a week

2.8.3 Preparation of trilayer scaffolds combined with skin explants.

A sterile No. 22 scalpel was used to cut sterilized trilayer electrospun scaffolds into 15 x15 mm^2 pieces. These were placed into 6 well plates with stainless steel metal rings were on the top. To this, the prepared skin explants, suspended in 0.5 mls of 10% serum Green's Media was added to the centre of each metal ring. 2 mls of 10% serum Green's media was used to top up the surrounding scaffold outside the steel ring and these were incubated at 37°C, humidified 5% CO_2 for 48 hours. At the end of this time period, the constructs were raised to an air liquid interface using the metal grids to support the skin explant seeded scaffolds. These were then cultured for 7, 14 and 21 days respectively with media changes using 10% serum Green's three times a week.

2.8.4 Preparation of skin explants on trilayer scaffolds with varying concentrations of fibrin and Tisseel (Baxter, Deerfield, IL, USA)

A sterile No. 22 scalpel was used to cut sterilized trilayer electrospun scaffolds into 15 x15 mm^2 pieces. These were placed into 6 well plates to this, the prepared skin explants, suspended in 0.5 mls of 10% serum Green's Media was added. This was followed by adding 30µml of fibrinogen (concentrations 18.75mg/ml, 67mg.ml and 86.5 mg/ml) and 10µml of thrombin (concentrations 2.5 I. U, 4.5 I. U, 6.5 I. U) to create a fibrin clot, which was mixed with the explants to allow even distribution. For experiments with Tisseel (FDA approved product for clinical use consisting of fibrinogen and thrombin in concentrations that combine to give a dense fibrin clot-see chapter 6 for further details), this was allowed to reach room temperature over a two-hour period before assembling the kit, which facilitated simultaneous ejection of fibrinogen and thrombin onto the skin explants. Following this, 2 mls of 10% serum Green's media was used to top up the surrounding scaffold these were incubated at 37°C, humidified 5% CO_2 for 48 hours. At the end of this time period, the constructs were raised to an air liquid interface using the metal grids to support the skin explant seeded scaffolds. These were then cultured for 7, 14 and 21 days respectively with media changes using 10% serum Green's three times a week.

2.8.5 Preparation of split thickness skin with variable dermal thickness to determine its effect on outward skin cell migration

Skin of variable dermal thickness were prepared by altering the settings of the Watson knife that was used to create the split thickness skin. Once the skin was harvested, it was cut into 5x5 mm2 pieces and a digital micrometer was used to determine the thickness of the sample. The epidermal thickness was assumed to be the same, as all samples for this experiment came from the same patient using the same block of skin. The squares of skin were finely minced into 16 smaller pieces and placed in the centre of a 6 well plate. 2 mls of 10% serum Green's media was added and samples were incubated at 37°C, humidified 5% CO_2 for 7 days. Each day, the skin cells were stained with Rose Bengal and the distance of the stain from the edge of the skin explants were plotted against the thickness of the skin.

2.8.6 The sandwich technique- placing skin explants between two squares of electrospun scaffold to aid cellular migration.

Templated PHBV/PGLA (75:25) trilayer electrospun scaffolds were prepared as above in addition to skin explants. The scaffolds were placed into 6 well plates. To this, the prepared skin explants, suspended in 0.5 mls of 10% serum Green's media was added to the centre of the scaffold. Another layer of trilayer electrospun scaffold was placed on top of the skin explants followed by a stainless steel ring to hold the construct in place. 2 mls of 10% serum Green's media was used to top up the surrounding scaffold outside the steel ring and these were incubated at 37°C, humidified 5% CO_2 for 48 hours. At the end of this time period, the constructs were raised to an air liquid interface using the metal grids to support the skin explant seeded scaffolds. These were then cultured for 7, 14 and 21 days respectively with media changes using 10% serum Green's three times a week.

2.8.7 Preparation of skin explants with fibroblast co-cultured electrospun scaffolds with and without fibrin.

A sterile No. 22 scalpel was used to cut sterilized trilayer electrospun scaffolds into 15 x15 mm^2 pieces. These were placed into 6 well plates followed by stainless steel metal rings placed on top. To this, 1x10^5 CMFDA fluorescently labelled fibroblasts (between passage 4 to 9) were placed in the centre of the metal ring suspended in 0.5mls of 10% serum DMEM. 2 mls of DMEM was used to top up the surrounding scaffold outside the steel ring and these were incubated at 37°C, humidified 5% CO$_2$ for 48 hours. The constructs were then removed from the incubator and the presence of fibroblasts were verified by observing for CMFDA. The media was discarded and the electrospun scaffolds were turned over. New sterilized stainless steel metal rings were then placed onto the centre of the square pieces of fibroblast seeded electrospun scaffolds and skin explants prepared as above, were placed in the centre of the ring. For experiments including fibrin, 30 µml of 18.75mg/ml of fibrinogen was placed in the centre of the skin explants followed by 10µml of 2.5 I. U of thrombin. Once the fibrin clot set, 2mls of 10% serum Green's media was placed in the well. These were incubated at 37°C, humidified 5% CO$_2$ for 48 hours. At the end of this time period, the constructs were raised to an air liquid interface using metal grids to support the skin explant seeded scaffolds. These were then cultured for 7, 14 and 21 days respectively with media changes using 10% serum Green's three times a week.

2.9 Outcome Measurements

2.9.1 Cell Tracker ™ Fluorescent Probes

CMFDA and CMTPX (Invitrogen, USA) were used to stain fibroblasts and keratinocytes respectively to ensure that they were present on scaffolds before they were raised to an air liquid interface. These are non-toxic probes that monitor cell movement, location and proliferation. They cross the cell membrane and stay within that cell for several daughter generations. They display florescence for at least 72 hours to a maximum of 7days. Fibroblasts were stained with CellTracker green. Flasks containing fibroblasts were removed from the incubator and media discarded, followed by three washes of sterile PBS. CMFDA 10 mM was prepared by adding the required amount to DMSO. This was then added to 10 mls of 10% serum free DMEM. The flasks were then incubated at 37°C, in a humidified atmosphere of 5% CO2 for 45 minutes. After this time the media was discarded followed by three washes of sterile PBS. 3mls of Trypsin/EDTA was then added to each flask and once again incubated at 37°C, in a humidified atmosphere of 5% CO_2 for 10 minutes until cells were detached from the bottom of the flask. The cell/Trypsin EDTA suspension was then placed into a sterile 30 ml universal pot together with 7 mls of 10% serum DMEM to

stop the action of trypsin. This was then centrifuged at 200g (1000 rpm) for 5 minutes. The supernatant was discarded and the cell pellet resuspended in 5 mls of 10% serum DMEM. The cells were counted using the standard method. The media was then adjusted to ensure that there were 1×10^5 fibroblasts per 0.5 ml which was the number used for seeding onto scaffolds. The labelling of keratinocytes followed the same method. CMTPX was used at a concentration of 10mM. The cells once labelled were harvested by the same method and resuspended in the required amount of serum 10% Green's media to ensure that a concentration of 3 $\times10^5$ cells was present in 0.5 mls of media. Once seeded the cells could were imaged in Olympus IX73 Epifluroscent Microscope (KeyMed Medical & Industrial Equipment Ltd. Essex, UK) at 570 nm λ_{ex}–620 nm λ_{em} (CellTracker red) and 480 nm λ_{ex}–533 nm λ_{em} (CellTracker green).

2.9.2 Alamar Blue^{TM} assay (AbD, serotec, Kiddlington, UK).

This is a quantitative, non-toxic assay that can be performed during the time course of an experiment, as it does not result in cell lysis. The active reagent is a blue dye called resuzarin, which is cell permeable and non-fluorescent. Upon entering the cells it is reduced to resofurin, which produces a bright red/pink fluorescence (Figure 2.6). Viable cells continuously reduce resuzarin to resofurin

thereby the amount of florescence produced is proportional to the number of living cells in the sample. This generates a quantitative picture of cellular viability.

Samples were washed three times with sterile PBS. Alamar Blue powder was weighed out and dissolved in PBS to obtain a 10% dilution. A filter was then used to sterilize the solution. 10% Alamar Blue solution was added to constructs to ensure that they were completely covered with the dye. The plates were then incubated for 60 minutes at 37°C, in a humidified atmosphere of 5% CO_2. At this time, it was usual to observe some colour change in the samples, confirming cellular viability. 200μl of dye from each sample was pipetted into a labelled 96 well plate. They were then read at 570nm (manufacturer's instructions) in a colorimetric plate reader (BIOTEK, NorthStar Scientific LTD, Leeds, UK). This was performed at various time checkpoints for each experiment. The data was analysed to obtain % absorbance.
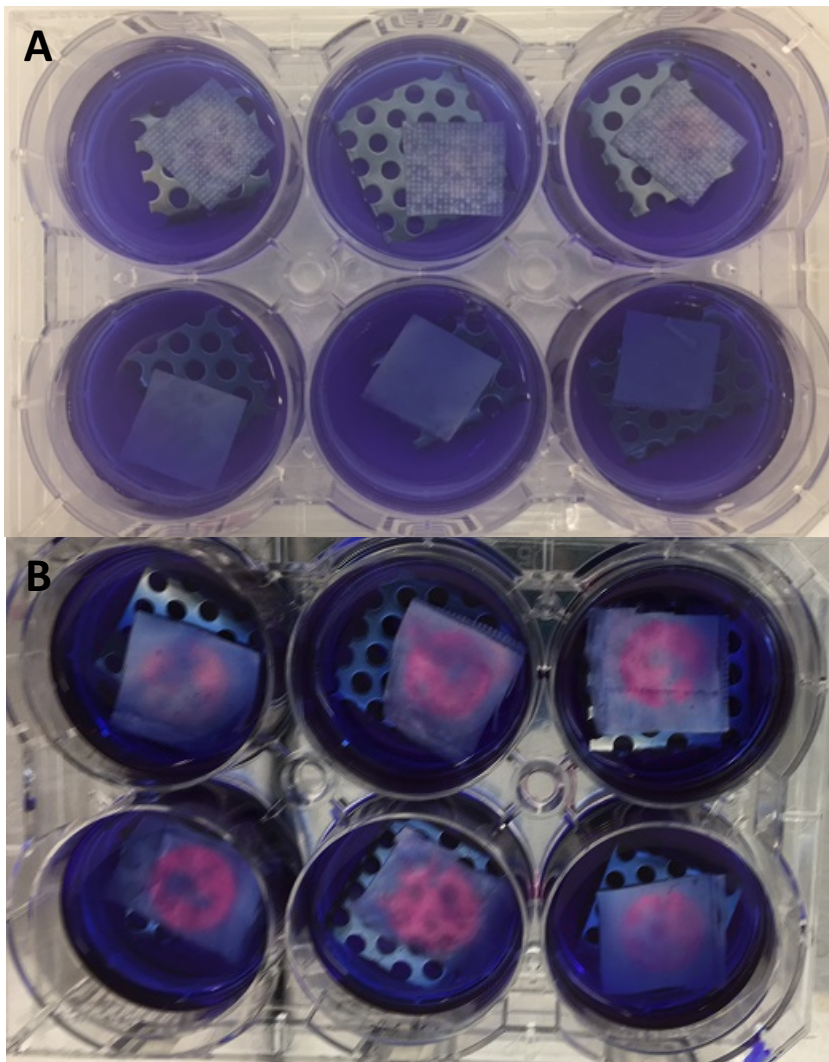
Figure 2.6: *Alamar Blue cellular viability assay. A: Pre incubation cell seeded samples. B: Post incubation cell seeded samples*

2.9.3 Rose Bengal Staining

Cellular outgrowth from skin explants onto scaffolds or on tissue culture plastic were assessed using 1% Rose Bengal (Sigma Aldrich, Dorset, U.K) for 5 min. The samples were washed several times with PBS to remove any excess Rose Bengal. These stained areas were then photographed to obtain an image that was

assessed quantitatively using Image J (NIH, USA) to estimate the extent of outgrowth from explants onto the membranes. The difference in the areas were averaged and the data expressed as mean ± SEM.

2.9.4 Fixation of Constructs

This was done using 3.7% Formaldehyde made by adding 10 PBS tablets to 900ml of water followed by 100ml of stock 37% formaldehyde (Sigma-Aldrich, Dorset, UK). Prior to fixation, media was discarded from each construct and they were washed three times with sterile PBS. To each well plate 3.7% formaldehyde was added to ensure that each construct was completely submerged in the liquid. This was incubated for at least 2 hours at 4°C before conducting further tests or stored at 4°C until required.

2.9.5 DAPI Staining

DAPI (4', 6-diamindino-2-phenylindole dihydrochloride) (Sigma-Aldrich, Dorset, UK) is a blue fluorescent stain that preferentially binds to nuclear DNA. This produces a x 20-fluorescence enhancement once bound to the DNA. It penetrates the cell membrane optimally if the cells are fixed therefore all

constructs were fixed using 3.7% formaldehyde as above before performing the stain. Stock vials of DAPI were made to a concentration of 1mg/ml by diluting in DMSO and aliquots of 10μl were made. These were then diluted in 10mls of sterile PBS to obtain a final working concentration of 1μg per ml. This was then added to each construct just enough to cover it completely. It was then incubated at 37°C, in a humidified atmosphere of 5% CO_2 for 45 minutes. The excess DAPI was then discarded and each construct was washed three times with sterile PBS. The presences of the stain in the constructs were studied using the Olympus IX73 Epifluroscent Microscope at λ_{ex}385nm/λ_{em}461nm imaging along the whole length of the scaffold strips.

2.9.6 Total Collagen Quantification: Sirius Red staining

This is both a quantitative and qualitative outcome measure. It is assumed that functional fibroblasts will produce collagen on the constructs. Sirius red is a well-known stain that is used in collagen histochemistry. It binds to and stains all forms of collagen fibrils thereby quantifying the amount of collagen in a given area of the construct. 0.1% Sirius red stain (Direct red 80, Sigma-Aldrich, Dorset, UK) was prepared by weighing out the desired quantity and adding it to saturated Picric Acid 0.9-1.1% (Sigma-Aldrich, Dorset, UK).   This stain was performed on fixed constructs. The 3.7% formaldehyde was removed from each

well plate and the samples were washed three times with sterile PBS. Sirius red was then added to each well plate ensuring that the constructs were completely covered by the stain. They were placed onto a shaking platform and left overnight. The following day, the constructs were washed at least three times in distilled water to remove any excess stain. They were then left to air dry in a laminar flow hood for 48 hours. Each construct was weighed and this was recorded. Following this 3mls of 0.2M NaOH: Methanol 1:1 solution was added to each well plate to elute the absorbed Sirus red and placed on the rocking platform for 15 minutes to allow the methanol to evaporate. 200µl of the eluted dye was then pipetted into a new 96-well plate. The absorbance was then read at 490nm in a spectrophotometer (BIOTEK, NorthStar Scientific LTD, Leeds, UK). The absorbance of stain per gram of dry construct was calculated. Furthermore, the difference between absorbance per gram of constructs with seeded cells or explants and those with no seeded cells or explants were calculated to assess the amount of collagen deposited by functioning cells on scaffolds.

2.9.7 Histology

Samples after being fixed in 3.7% formaldehyde were cryopreserved prior to staining with Carazzi's Haematoxylin and Eosin Staining. This method was

chosen, as it does not result in destruction of the scaffold compared to wax embedding. These stains were chosen to highlight the cellular architecture/ location within the scaffold. Haematoxylin is a dark blue/violet stain that is alkaline in nature and stains cellular nuclei blue. Eosin is a pink acidic stain that binds to cytoplasmic structures resulting in shades of red, pink and orange. Together they give an indication of the location of cells within the scaffold and any protein/ extracellular matrix produced by viable cells eg. keratin and collagen.

2.9.7.1 Cryopreservation

The 3.7% formaldehyde was removed from the samples and they were washed three times with sterile PBS. Samples were then placed in a plastic cassette and embedded with Optimal Cutting Temperature O.C.T ^{TM} (Tissue-Tek® 4583, Sakura Finetek, Netherlands, Europe) compound.

A pair of metal forceps was used to gently and slowly submerge the plastic cassette in liquid nitrogen (-197°C). Once the sample was completely frozen, it was removed from the liquid nitrogen and the plastic cassette very gently. A sterile No. 22 blade was used to cut the sample in half. It was then fixed to mounting device the O.C.T as an adhesive and left to solidify in the cryostat (Leica CM1860 U.V, Leica Biosystems, Germany). The sample was then sectioned

using a cryostat into 7μm slices and mounted on frosted labelled glass slides (Fisher Scientific, Loughborough, UK) and left to air-dry overnight.

2.9.7.2 Haematoxylin and Eosin Staining

The slides were soaked in distilled water for 2 minutes before they were stained with Harris Haematoxylin (Sigma-Aldrich, Dorset, UK) for 1.5 minutes. After two washes in running tap water for a total of four minutes, the slides were then stained with eosin (Sigma-Aldrich, Dorset, UK) for five minutes. The samples were then washed in tap water again for 1.5 minutes and then dehydrated through 70% alcohol (IMS; Industrial Methylated Spirit, Fisher Scientific, UK Ltd.) by dunking. This was followed by 100% alcohol for 30 seconds and finally, they were cleaned in two changes of xylene (Fisher Scientific, UK Ltd) lasting one minute each. They were then mounted with a coverslip using a DPX mounting medium (Fisher Scientific, UK Ltd.) and left to air-dry overnight. The following day images were taken of each slide using a light microscope: Motic B5 Professional and the software used for analysis was Motek Images Advanced 3.2 (Motek CME group ltd). Scale bars and final adjustment of the images were performed using ImageJ software (US National Institutes for Health, NIH Bethesda, Maryland).

2.10 Immunohistochemistry

Electrospun scaffold cultured with isolated cells and co-culture with skin explants were assessed in both 2D and 3D culture immunostaining using Antibodies against proliferating cells (Ki67), keratinocytes (pancytokeratin) and collagen (collagen IV).

Templated and non-templated trilayer PHBV/PGLA 75:25 and monolayer PGLA 75:25 electrospun scaffolds that were cultured with isolated fibroblasts and keratinocytes for 14 days at air liquid interface were fixed in 3.7% formaldehyde for at least 30 minutes. They were then cryopreserved using the method as outlined above. Slices of 7 μm were made onto glass slides and left to dry overnight. Immunostaining was also performed on fixed 14 day samples where fibroblasts were used to culture the templated trilayer electrospun scaffolds before adding skin explants. Due to the 3d nature of these scaffolds, we elected to immunostain the entire fixed sample followed by confocal microscopy to analyse the resulting images. Once these samples were fixed with 3.7% formaldehyde for at least 30 mins, the fixative was removed and samples were washed with PBS three times. The method from this point is the same for both sample preparations. 100% ethanol was added to samples for two minutes and left to air dry at room temperature. Following this, the samples were incubated with Triton X100 (0.25%) for thirty minutes. After three washes of PBS,

quenching was performed using ammonium chloride (50mM) for ten minutes. This was followed by adding fish gelatin (0.2%) as a blocking buffer for 30 minutes. Samples were then incubated overnight using the following dilutions:

- Ki67- monoclonal antibody (e fluoro 570; eBioscience, ThermoFisher Scientific, Renfrew); stock 0.5mg/ml unconjugated; Cat 14.5698.82. 1 µml of the antibody was added to 1000 microml of fish gelatin(0.2%) to obtain a dilution of 1 in 100.

- Pancytokeratin (ThermoFisher Scientific, Renfrew) - monoclonal antibody AE1/AE3 Alex fluro-488; e bioscience; stock 0.5mg/ml (conjugated). Cat 53.9003.82. Dilution used was 1 in 300.

- Collagen IV (Alexa Fluor 488, e Bioscience, Thermofisher Scientific, Renfrew); stock 0.5mg/ml (conjugated). Dilution used was 1 in 100.

The following day samples were washed three times with PBS and DAPI was added (1 :1000 dilution in PBS) for 30 minutes. The samples were then rehydrated and dehydrated by adding distilled water for 10 minutes to samples followed by 100% ethanol for one minute. Samples that were prepared on glass slides were then mounted using a coverslip and DPX mounting medium and left to air dry for two hours in the dark.

2D images were obtained using an Olympus IX73 Epifluroscent Microscope using the following excitation and emission wavelengths:

- DAPI: λex 385 nm/λem 461 nm.

- TRITC: λex 545/25 nm/λem 605/70 nm.

- FITC: λex 488 nm/λem 519 nm.

2.11 Scanning Electron Microscopy

This was used to visualize scaffolds before and after seeding cells and skin explants. Furthermore, it was able to demonstrate the presence of the 3 distinct layers of polymers and stem cell niches templating on the scaffolds.

2.11.1 Sample Preparation

All samples containing cells and skin explants were fixed in 3.7% formaldehyde. After three washes in PBS, 2 mL 0.1 M cacodylate buffer was added and incubated for 5 min. This was followed by 2 mL 2.5% gluteraldehyde in distilled water and incubated for 30 min.

The gluteraldehyde was removed and 2 mL of cacodylate buffer was again added to rinse any remaining gluteraldehyde. 500 μL of osmium tetraoxide was then

added and incubated for 2 hours. The osmium tetraoxide was then removed and 2 mL cacodylate buffer added and incubated for 15 min. Subsequently the samples were incubated for 15 min each with 75, 95 and 100% ethanol and freeze dried for 16 hours.

2.11.2 Sample Imaging

Samples were cut into $2x2mm^2$ square pieces and mounted onto 12.5mm stubs (Agar Scientific, Stanstead, UK) using carbon conductive tape.

They were first painted with silver dag (Agar Scientific, Stanstead, UK) and then sputter coated with gold for 2 minutes at 15 mA current (Gold coater; Edwards sputter coater S150B, Crawley, England). Samples were imaged using a scanning electron microscope (Inspect F, FEI, Netherlands, Europe) at an accelerating voltage of between 10-15 kV and a SPOT size of 3.

2.12 Statistical Analysis

All tests that resulted in quantitative data, P values are calculated for differences between samples using a two-sample t-test with no assumption of equal variance.

Chapter 3

Design and Production of Candidate Scaffolds for Dermal Replacement

3.1 Introduction

The aim of this chapter is to detail the design and production of an electrospun biodegradable scaffold designed to be used with finely dissected pieces of split thickness skin as a one stage approach to full thickness skin defect reconstruction. The scaffold is designed as a trilayer, templated membrane like structure with the aim of encouraging fibroblast entry into the lower dermal layer and keratinocyte production of an epithelial barrier layer separated by a pseudo-basement membrane. Human skin cannot be viewed simplistically as two layers (Figure 1.2), it is a complex homeostatic system in which its anatomy plays a significant role in its ability to continuously regenerate, be stable when subject to shear forces and most importantly maintain its barrier function.

The basement membrane to recap, is a thin layer of specialized extracellular matrix onto which the epidermis is attached. It links the epidermis to the dermis and provides a barrier against epidermal migration(4). It is morphologically distinguished into four layers: the plasma membrane of the basal keratinocytes; lamina lucida; lamina densa and sublamina densa. These layers augment the keratinocyte to dermis attachment(5). The lamina densa provides mechanical strength and the middle layer that forms a sheet like structure comprising of type IV collagen, nidogens and perlecan(7). The sublamina densa (lamina fibroreticularis) is the deepest layer of the basement membrane which contains

collagen VII that extends through fibrils and insert into plaques in the reticular dermis(8).The basement membrane is topographically organized into peaks and troughs in post-natal skin. These develop mid-gestation where prior to this; the dermal epidermal junction is flat(9).These structures referred to as rete ridges, range from 50-400 micrometres in width and 50-200 micrometres in depth(10),(11) Epidermal stem cells lie in specific locations relative to the rete ridges depending on their location in the body. The rete ridges are an important feature in the topography of the dermal-epidermal junction. It provides structural integrity for the epidermal-dermal interface and defines the cellular microenvironments, which then determines the functioning of the basal and suprabasal keratinocytes in these regions(14).

To achieve structures which would support epidermal keratinocytes and dermal fibroblasts and yet keep them separated while securely attached to each other we designed a trilayer structure. This was composed of two layers of microfibres of biodegradable PGLA (75:2%) separated by a very thin layer of nanofibrous PHBV. The latter was designed so that cells could attach to these fibres but the spaces between them were so compact that cells could not migrate through them. In producing this trilayer it was also necessary to produce the three layers so that they did not delaminate when cells were placed on them. Further the

nano fibre layer was also spun over a template designed to mimic the folds of the rete ridges. This is illustrated in cartoon form in Figure 3.1
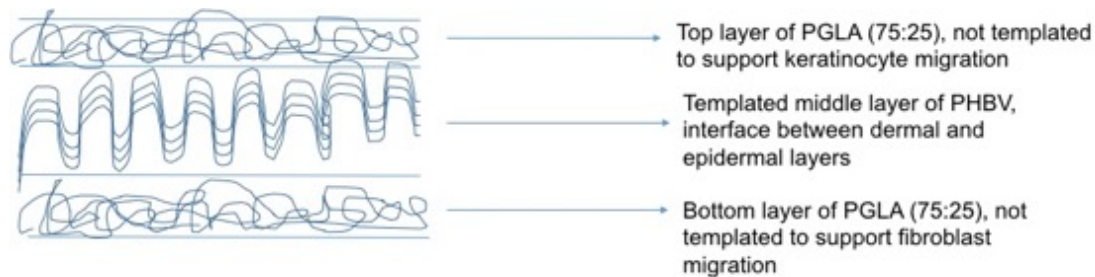
Figure 3.1: *Cartoon demonstrating structure of the templated trilayer of nanoporous PHBV between two layers of microporous electrospun fibres.*

PGLA (75:25*)* fibres were spun to be microfibres with relatively large pores between them to allow cell entry while PHBV were spun as nanofibres with relatively compact pores between them to prevent cell migration through this layer. The ability to achieve microfibers using PGLA (75:25) and nanofibres using PHBV to create this trilayer structure was established by Bye et al(114), not only by using different polymers but also by varying the distance between the tip of the needle and the collector (10cm for PHBV and 17cm for PGLA 75:25).

PGLA (75:25) is a linear amorphous co-polymer composed of poly (lactic acid) PLA and poly (glycolic acid) (PGA). They belong to the group of saturated poly (α-hydroxy esters). These polymers degrade via hydrolysis through de-esterification. Under physiological conditions PGA is a rigid thermoplastic

material with a high crystallinity (46-50%) as a result it is not soluble in most organic solvents. It has glass transition and melting temperatures of 36°C and 225 °C respectively(151). PGA undergoes hydrolysis and may also be degraded by surrounding enzymes that have esterase activity. The breakdown product is glycolic acid, which then enters the tricarboxylic acid cycle. Following this it is excreted as water and carbon dioxide. This makes PGA a very attractive option for clinical applications as its degradation products are non-toxic and conform to a known excretion pathway.

Poly (lactic acid) (PLA) is present in three isomer forms d (-), (l+) and racimi (d.l). The d, l form is a popular source of polymer for drug releasing scaffolds. PLA is hydrophobic, semi-crystalline and has a glass transition temperature between 60-65°C and a melting temperature of around 170 °C. It degrades via hydrolysis into lactic acid, which then enters the Krebs's cycle(152).

PGLA can be prepared using various ratios of the constituent monomers i.e. lactic acid (LA) and glycolic acid. PGLA is therefore identified by its constituent monomer ratios. Therefore, PGLA (75:25) reflects a copolymer consisting of 75% lactic acid and 25% glycolic acid. This ratio strongly influences the physical and chemical properties of the resultant co-polymer. PGLA is advantageous over its constituent monomers in that it dissolves in a wider range of solvents including

chlorinated solvents and acetone. PGLA is available in two forms, which is dependent on the isomer of the lactide that it is made of (D, L and L, D). The PGLA used in this thesis was of the (D, L) form.

PHBV is a biodegradable co-polymer made from units of hydroxybutyrate with anywhere between 0 and 24% of hydroxyvalerate units appearing randomly throughout the polymer chain(153). Polyhydroxybutyrate (PHB) is produced in nature by a variety of bacteria, which store it as a source of carbon and energy. However, this material itself is brittle and has deficiencies in thermal stability, which limits its ability to be used as a manufacturing material. The addition of polyhydroxyvalerate to the polymer chains leads to a reduction in melting point and crystallinity of the copolymer and enables milder processing conditions to be used(154). PHBV can be manufactured by fermentation of a wide range of microorganisms. The PHB is produced during the first fermentation process and PHBV is obtained when the bacteria are fed a controlled amount of organic acid during a second stage of fermentation. Once the polymer is made it is then separated from bacterial cells using solvent extraction after breaking down the bacterial cell wall(153). PHBV is more stable than polyesters and is more resistant to hydrolytic degradation(155). This property can be exploited in a variety of biomaterials that require longer periods of mechanical stability e.g. bone regeneration. It was selected as a polymer to make the pseudo-basement

membrane because of its biocompatibility, its ability to be reproducibly spun into nano fibres and it's known route of breakdown. It provides a large surface area of nanofibres for extra cellular matrix deposition and degrades at a slower rate than PGLA, which provides a small amount of additional support for proliferating cells during the remodelling phase of wound healing.

Electrospinning is one of several methods that are used to produce porous 3D biodegradable scaffolds for tissue-engineering applications. Other fabrication techniques have been well described. These can be divided into those that use porogens in biomaterials (solvent casting, particulate leaching, gas foaming and freeze-drying and phase separation), solid free-form or rapid prototyping technologies (selective laser sintering, stereolithography and 3D printing) and techniques that develop woven and non-woven fibres (use of thermal energy or adhesives to result in a porous fibrous network)(156). Electrospinning is our chosen technique to produce these porous scaffolds, as it is cost effective, reproducible and easily up scaled to increase production for wide spread clinical use. Furthermore, it provides desirable scaffold architecture with a large surface area for cellular attachment, migration and proliferation that can closely mimic the collagen scaffold of the dermis.

3.2 Properties of a dermal scaffold

Any scaffold for tissue engineering applications for human use must have certain features that make it compatible for use in patients. These can be divided into essential and desirable. All scaffolds at a minimum must be biocompatible. They need to be safe for use in man with negligible inflammatory and immunological reaction from the host(157). Scaffolds should also act as a support matrix for cells to adhere to and function to regenerate the underlying tissue(158). Once the patient's own cells have regenerated the tissue components the scaffold should degrade into known by products that are excreted via established pathways. Scaffolds in tissue engineered applications aim to be temporary support matrices as opposed to a permanent implant. By modifying the choice of polymers used in production of the scaffold then scaffolds can be designed to degrade within a time frame to match the expected regeneration period of that tissue for example in skin this would ideally be at the 2-3 month mark when the wound is in the remodelling phase, and the patient's own collagen is being remodelled from type 1 to type 3. Furthermore, the scaffold should have similar or close enough mechanical properties to accommodate the deformational forces that are expected to be present in the tissue into which it is being implanted. The different parts of the skin are also expected to experience varying mechanical forces. Geerligs et al. (159) found the Young's modulus for the epidermis to be 1.1 ± 0.2 MPa and

Crichton et al (160)reported the Young's modulus of dermis to be 7.33–

13.48 MPa.

This mechanical integrity should last from implantation to integration-essentially for as long as it is required(161). Importantly also scaffolds should be able to withstand surgical manipulation and handling and the deforming forces that will occur during the implantation process.

In order for scaffolds to act as a structural support for tissue regeneration the underlying architecture is of great importance. Scaffolds should possess an appropriate porosity that facilitates cellular penetration and diffusion of nutrients and oxygen to facilitate cellular proliferation. Furthermore porosity is also important in ensuring that waste products of cellular metabolism are able to diffuse out of the structure(157). Additionally, apart from supporting cells and their by-products, they should also be able to support a vascular tree which is needed if the material is going to engraft successfully. These features outlined above can be considered as essential and need to be present to implant these materials into man.

Other features can be considered desirable, which can be directed to the tissue that the material is aiming to mimic. For skin tissue engineering, scaffolds made from natural polymers like collagen and chitosan naturally possess ligands that

are needed for cells to interact and attach to the fibres of the scaffolds. These ligands are often not present in scaffolds that are made from completely synthetic substances. These ligands can be incorporated into the scaffolds by means of protein adsorption, however even if they are not deliberately incorporated once a biomaterial comes into contact with any biological fluid eg blood or tissue fluid, proteins -primarily fibronectin -will begin to adhere to the fibres via protein adsorption. When cells are ultimately seeded onto the scaffolds the material will already have been coated in protein hence ultimately cellular attachment will depend on the affinity of the cells to bind to the dynamic protein layer(162).

Moreover in order for a scaffold or any tissue engineered construct to become clinically and commercially viable it needs to be cost effective and should be able to be seamlessly up scaled from single unit to batch production(163). This is the ideal and it is appreciated that many tissue engineered applications will have a significant cost attached to production for clinical use however, keeping costs to a minimum will always be desirable especially for widespread clinical uptake in developing countries. Another critical issue in the manufacturing process is the shelf life, storage conditions and delivery of the scaffold to the clinic such that there is no change to the architecture and integrity of the scaffolds.

For use in the clinic it is always desirable to have an off the shelf product, which is available as and when needed. It has to be noted that clinicians use biomaterials on a case by case basis which, in the case of traumatic wounds or burn injuries are unpredictable. This dictates the need for flexibility(164).

Another key issue in tissue engineered applications is the source of cells that are used to populate the scaffolds. To be successful all scaffolds ultimately need to be populated with cells that are present in the tissue that they are trying to replace. This is so that the cells can proliferate and deposit the extracellular matrix that will allow structure to effectively regenerate and reform. These cells most commonly autologous (from the patient) or less commonly allogeneic (sourced from donor patients). When cells are sourced from the patient themselves, they need to be cultured onto the scaffolds until they become suitable for implantation. This requires time, clean room facilities and the expertise to undertake this process.

As a result, although this approach has been clinically successful even within this group (78) uptake of these autologous therapies has not been extensive. Furthermore using biomaterials that require animal sources either as part of the dermal scaffold eg porcine or bovine collagen or in the process of culturing cells (media contains serum obtained from fetal calves) still carries a small risk of infection and immunological responses which can be immediate or delayed for

years(165). This includes the risk of bovine spongiform encephalitis, which can be transmitted from the FCS which is a vital component of cell media. Although the serum is obtained from animals in countries free of the condition, these prion diseases can be challenging and difficult to detect(166). Furthermore, the use of porcine products in man (xenotransplantation) is associated with hyper acute rejection due to the presence of a cell membrane antigen called the Gal epitope(167).

Property	Essential	Desirable
Biocompatible, safe and non toxic	✓	
Inert, non carcinogenic and non immunological	✓	
Predictable degradation products excreted through known physiological pathways	✓	
Predictable and modifiable breakdown time	✓	
Matrix supports cellular migration and proliferation	✓	
Pore and fiber size to facilitate oxygen and nutrient diffusion and waste excretion	✓	
Mechanical properties similar to the tissue being replaced	✓	
Can be up scaled for production in large quantities		✓
Handles well for clinical use		✓
Off the shelf		✓
Cost effective		✓
Free of all animal products		✓
No requirement for cell culture to populate scaffolds		✓

Table 2: *Ideal and Desirable Properties of Electrospun Scaffolds for Dermal Replacement*

3.3 Developing a suitable electrospun scaffold for dermal replacement

The candidate scaffold used in this thesis to be evaluated as a dermal analogue is a continuation of the work by the MacNeil group in the field of skin

regeneration. Foundation work performed by Blackwood et al compared PLLA, PGLA 50:50, PGLA 75:25 and PGLA 85:15. In vitro degradation studies performed here revealed that the PLLA degraded the slowest with it being largely intact after one year and the PGLA 50:50 degraded the fastest within 2 weeks(124). Given that a healing dermal wound has most of its tensile strength between 60-90 days, it would be desirable for the scaffold to be structurally intact for this period of time. This was found to be the case for the PGLA 75:25, which lost half its strength at the 3-month mark. As a result, PGLA 75:25 was chosen to be the top and bottom layer of this scaffold, as it will support the migration and proliferation of both keratinocytes and fibroblasts. Building on this, work by Bye et al we proposed the use of a trilayer scaffold consisting of layers of PLA microfibres on either side of PHBV nanofibers that was successful in supporting the proliferation and segregation of fibroblasts and keratinocytes for tissue engineered applications(126).

A trilayer scaffold for a dermal replacement could provide a synthetic basement membrane that would provide a framework around which cells could be stimulated to produce an extracellular matrix. The polymer chosen for this is PHBV, which produced a scaffold which with pores large enough to allow fibroblasts to cross into the layer intending to be the dermis and later allow

signalling molecules to cross from the dermis into the epidermis which is essential for skin regulation.

Furthermore, as previously discussed, the dermal epidermal interface is not flat but has undulated regions where the epidermis projects deep into the dermis which is called the rete ridges. These develop mid gestation and prior to this the interface is flat[25]. The height and overall dimensions of the rete ridges vary according to skin location on the body, age, and any acquired or congenital proliferative disorders. For example, the height of the rete ridges declines as we age(169) and increases in disorders which as psoriasis which are associated with high epidermal turnover(170). Furthermore, the concentration of rete ridges varies depending on the anatomical location the skin that is being assessed. The palms and soles, which have different epidermal and dermal structures compared to the rest of the body, contains a higher concentration of rete ridges. Presumably this is due to the higher epidermal cells turnover, thicker epidermis and the need for a strong anchor between the epidermis and dermis to prevent shear as these two areas are subjected to the greatest number of external forces compared to any other skin location. Stem cells which renew the epidermal cells lie in clusters outside the rete ridges just where the epidermal basal layer is most superficial and closest to the skin surface(13). Previous products that have successfully translated into clinical practice for example, CEA sheets without

consideration for the microstructure of skin have worked to some extent as the stem cells self-organise into clusters and their numbers become auto regulated. However, these materials require cultured cells which demand the time and facilities for cells to become confluent before transfer.

Our model relies on the migration of skin cells from the cut edges of skin explants, which in our opinion should be optimised as much as possible. We hypothesize that by mimicking the microstructure of skin, we can encourage not only cellular migration but cellular adhesion and organization so as to improve take, a problem which is seen not infrequently with epidermal skin substitutes eg CEA sheets. CEA sheets that are in clinical use, requires enzymatic detachment of the sheet from the culture vessels prior to transplantation. This results in the loss of keratinocyte basement membrane proteins and integrins which are cell surface receptors which are responsible for the attachment of the sheet to the underlying wound bed(171). Moreover, when keratinocytes achieve confluence they rapidly become differentiated cells which is associated with alteration of their integrin expression, this may explain the poor take observed by CEA sheets(172).

Additionally, undulations in the electrospun scaffolds can provide the construct with stem cell niches which aims to direct the stem cells to organize into their clusters around the niches. Feeding on this the MacNeil group then undertook

successful work in using stereolithography to fabricate templated scaffolds for corneal regeneration to provide these stem cell niches –it was felt that this concept could be applied to a potential dermal analogue[29].

There is little data on the significance of the rete ridges as firstly they vary significantly between individuals and also between the anatomical area that they have been taken from. Furthermore, the most used animal model for skin research, the mouse, has no rete ridges. Tissue engineered skin made using the traditional techniques of seeding isolated keratinocytes onto decellularized dermis has the issue of the collapse of the dermal topography which leads to cells migrating into empty hair follicles, creating false rete ridges(174).

Many electrospun scaffolds have been evaluated for dermal replacement using a combination of natural and synthetic biodegradable polymers in addition to various adjuncts to the electrospinning process eg plasma treatment and electrical stimulation of the fibres to produce a candidate scaffold fit for skin regeneration. For example, Poly (ε-caprolactone) (PCL)/gelatin was shown to be able to support the migration and proliferation of fibroblasts when seeded on both sides of the scaffold. It was felt that seeding fibroblasts on two sides as opposed to one would increase the capacity of cells to be loaded into the scaffold structure and also engage cellular adhesion and proliferation(175). Polyurethane/ polyvinylidene electrospun scaffolds have also been evaluated

for skin regeneration. Fibroblasts cultured on these scaffolds showed enhanced migration, adhesion and secretion of collagen(176). Poly (L-lactic acid)-co-poly(ε-caprolactone) (PLACL) and PLACL/gelatin complexes were made and plasma treated to improve their hydrophilic properties. After seeding these scaffolds with fibroblasts it was shown that the expression and secretion of collagen were significantly increased in plasma-treated PLACL/gelatin scaffolds compared to PLACL nanofibrous scaffolds(177).

Other groups have recognized the value of the trilayer construct. For example, Lin et al used trilayer chitosan electrospun scaffolds to replicate the striation of full thickness of skin more accurately(178). They were able to create discernible dermal, basement membrane and epidermal layers which maintained their structure even after seeding with keratinocytes and fibroblasts.

Our scaffolds are novel in that we are evaluating the incorporation of features of the fine micro topography of the skin like basement membranes and rete ridges on cellular attachment, proliferation and migration. Moreover, the methods of production of these scaffolds are easily reproducible and convertible to small scale manufacturing for clinical applications. The methodology of producing templated scaffolds was recently published from the MacNeil laboratory, using expanded cells to assess the initial performance of these scaffolds(179).

3.4 Evaluation of the characteristics of PGLA (75:25) and PHBV scaffolds.

To undertake this, we assessed morphological, physical and mechanical properties of these polymers when spun into scaffolds.

Morphological properties included the assessment of pore and fibre diameter size using ImageJ software of the SEM images of the polymers that constituted the electrospun scaffold. This was undertaken for three different pictures of the same area of scaffold, measuring the pore and fibre size diameter for at least (n=100) fibres and pores (n=100).

Mean= 4.161 μm
SEM= 0.200 μm
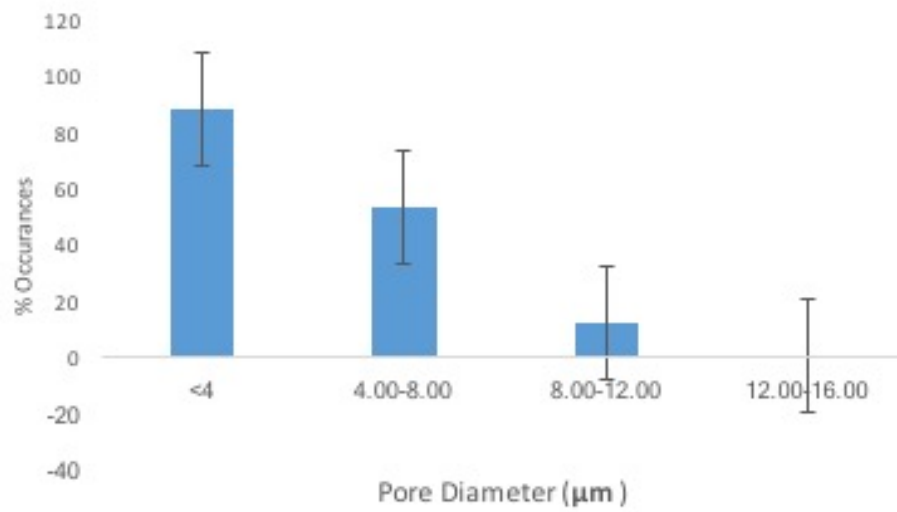
Mean= 1.89 μm
SEM=0.072 μm
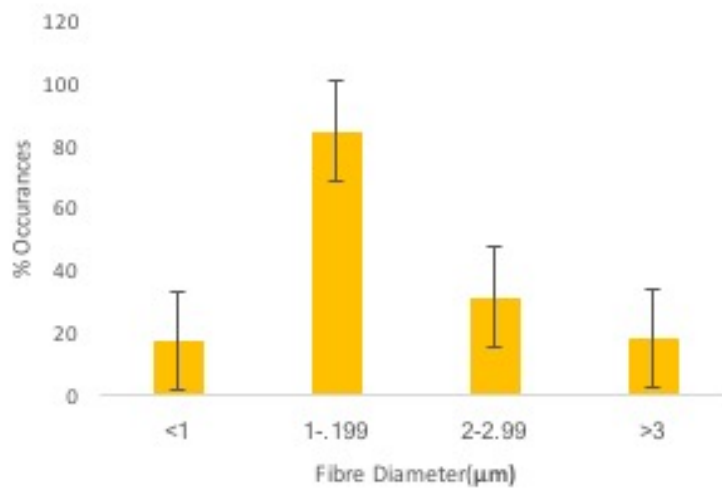
Figure 3.2a: *Pore diameter and fibre diameter distribution calculated from 100 fibres (n=3) for PHBV.*
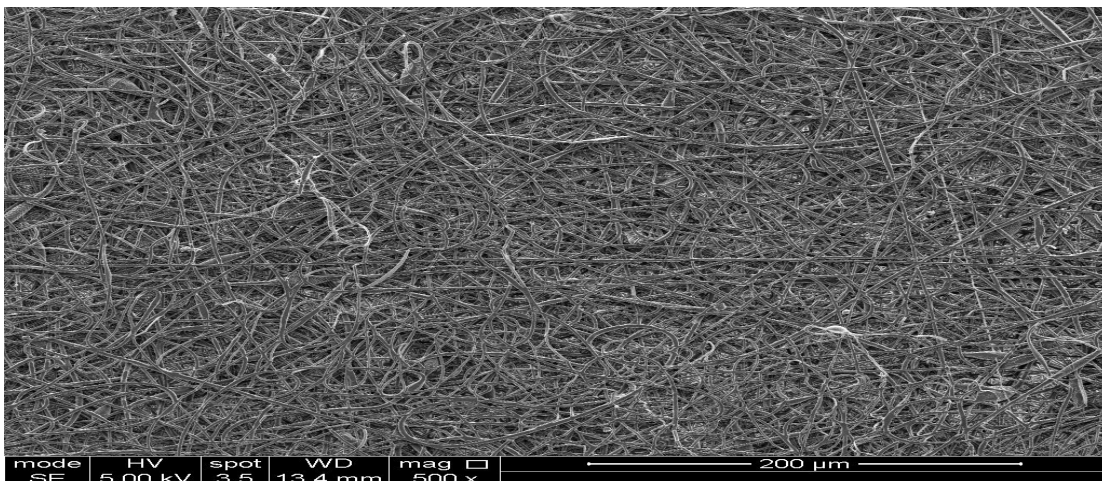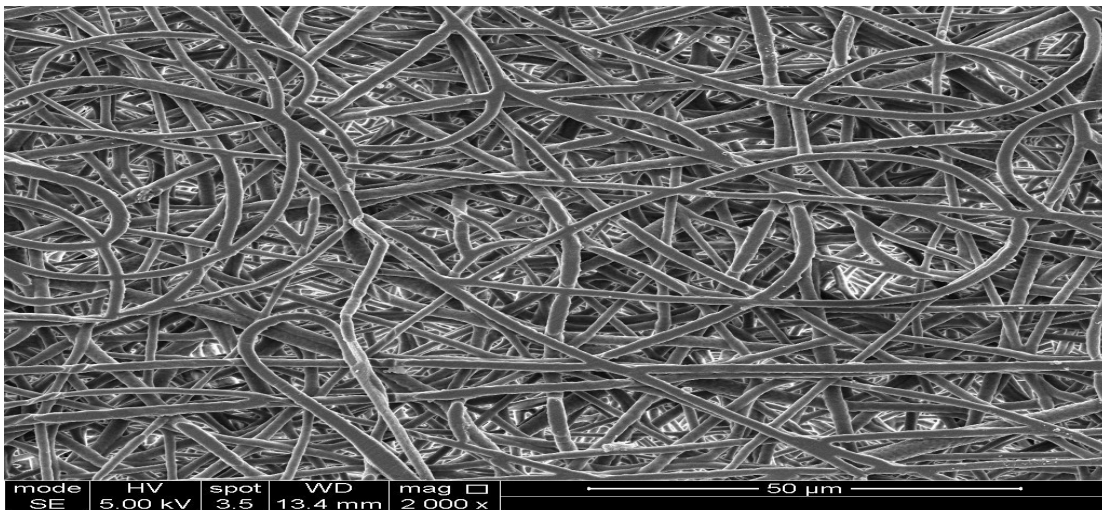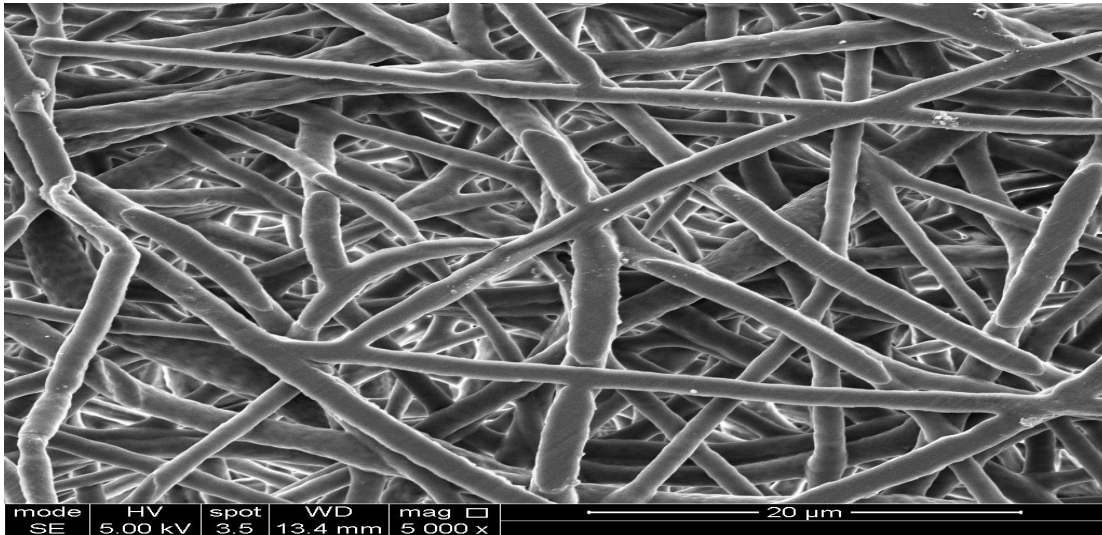
Figure 3.2b: *SEM images of PHBV scaffolds used in calculations.*

Mean = 8.062 μm
SEM =0.3035 μm
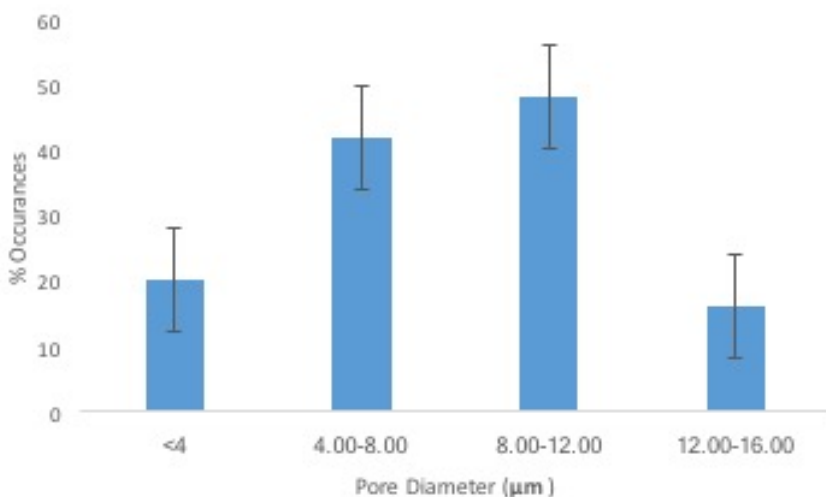
Mean = 2.22 μm
SEM = 0.074 μm
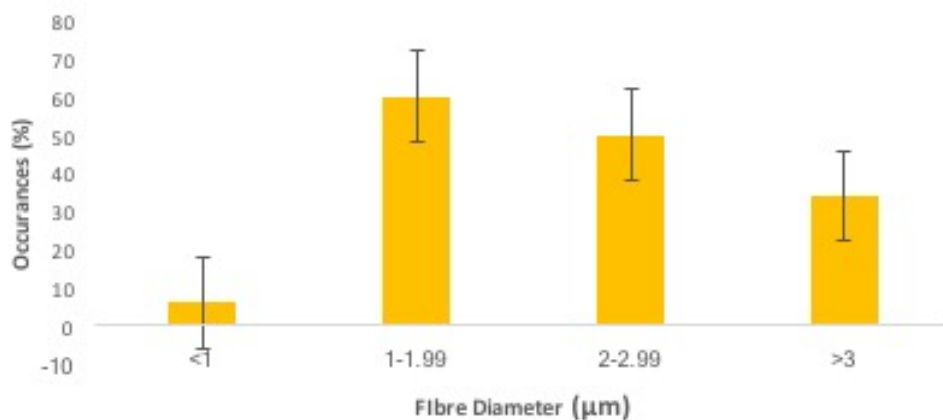
Figure 3.3a: *Pore diameter and fibre diameter distribution calculated from 100 fibres (n=3) for PGLA.*
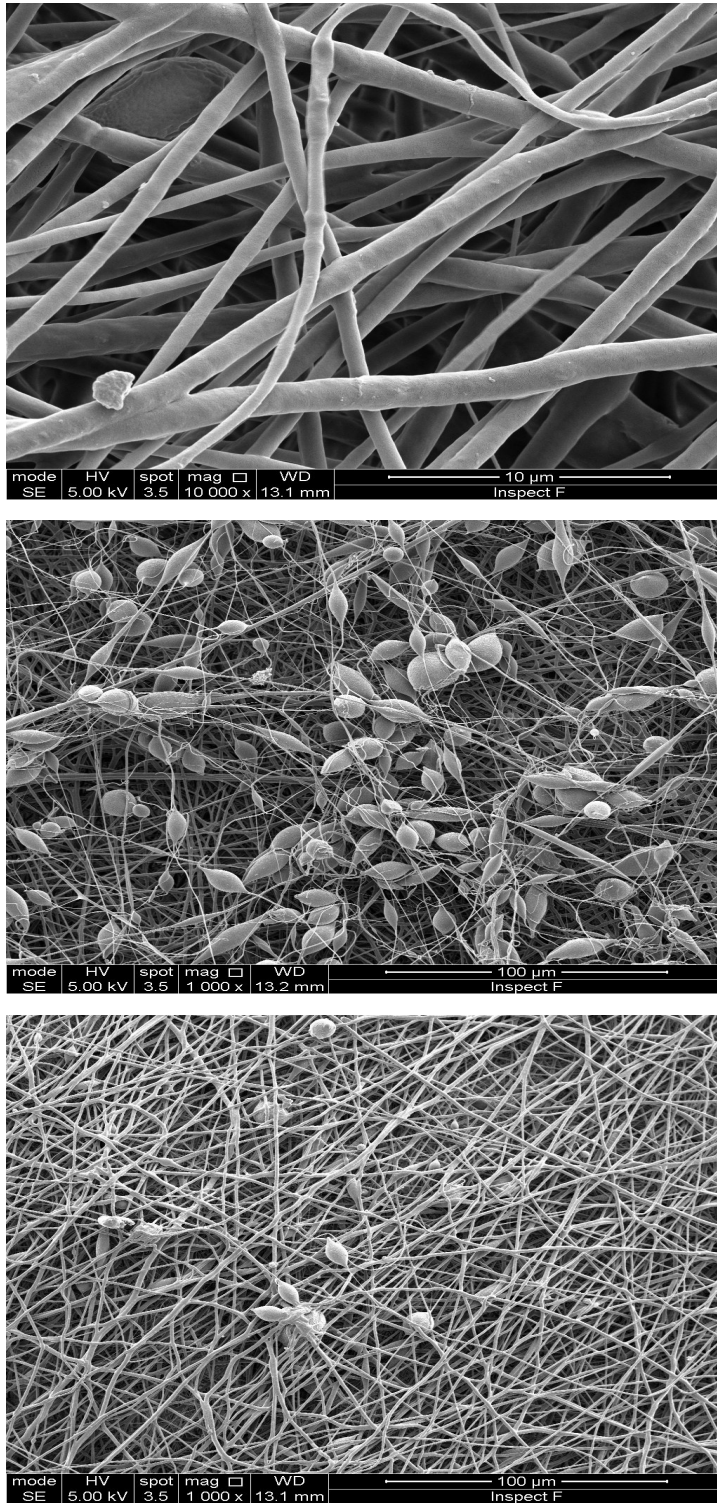
Figure 3.3b: SEM images of PGLA (75:25) scaffolds used to calculate pore and fibre diameter size

As shown in the SEM images, PHBV scaffolds consist of randomly orientated fibres with a mean diameter of 1.89 µm and with an average pore size of 4.161 µm (Figure 3.2). PLGA (75:25) scaffolds consisted of randomly orientated fibres with a mean diameter 2.22 µm of and a mean pore size of 8.062 µm (Figure 3.3). This difference is expected and reflects the design of the scaffolds. The PGLA (75:25) has larger fibres and pore sizes which facilitate the movement of keratinocytes and fibroblasts and has a large surface area for deposition of their bioproducts. PHBV has a smaller fibre diameter with smaller pores to mimic the basement membrane which although is not a defined anatomical structure, it is a distinct layer that separates the keratinocyte filled epidermis from the dermis but at the same time allow signalling proteins to move in between the two layers which is essential for development and renewal.

Scaffolds with pore sizes that are < 200 µm facilitate the development of vascular networks with small vessels at high density and shallow penetration which is suitable for skin substitutes as they are relatively thin compared to solid organ constructs(180). In line with this it is no surprise that clinically used dermal matrices like Integra and Matriderm have pore sizes of 20-125 µm and 20-150 µm respectively(181).

Mechanical properties of the scaffolds were measured using an uniaxial tensile test. The length, width (cut to be all the same) and thickness of all the samples

were measured to aid normalisation of results as these, if not constant would affect the mechanical properties measured. Parameters obtained from this test include the ultimate tensile strength (maximum stretch of the material), maximum elongation and the Young's modulus. This allows comparison of the biomechanical properties of our scaffold to skin itself, which is the material that we are trying to match as closely as possible. Furthermore, it is known that the mechanical properties of skin is affected by patient factors such as age, smoking and use of steroids, so for these measurements, samples were repeated from one patient. Due to patient confidentiality we were unable to map these factors onto the skin hence the use of one sample.

The Young's modulus (YM) is also known as the elastic modulus, as elongation of the material during this linear slope is known as elastic deformation. The material will recoil to its original size once the force stops. YM is the first linear slope when a material is stretched which is obtained from a stress strain plot. It reflects the relationship between stress (force per unit area) and strain (proportional deformation) in a material. It is calculated by dividing the tensile stress by the exertional strain in the elastic (initial linear) portion of the stress-strain curve that is obtained from the uniaxial tensile test. The steeper the slope, the stiffer the material. Once this portion of the curve is past, all further elongation observed is plastic deformation. Another parameter often measured

is the Ultimate tensile strength (UTS) which is the maximum strength of the material and the strain at ultimate tensile strength which is the maximum elongation of the material before it starts to snap.

All samples assessed in this project for the uniaxial tensile test were initially measured for size. The stress strain data for each sample was normalized before plotting the three above parameters described: YM, UTS and strain at UTS. This was done by dividing the stress by the area of the scaffold (width of scaffold x thickness) and dividing the strain by the length between the two grids on the tensiometer (effectively the length of the scaffold) to obtain a % of elongation from its original length.

A
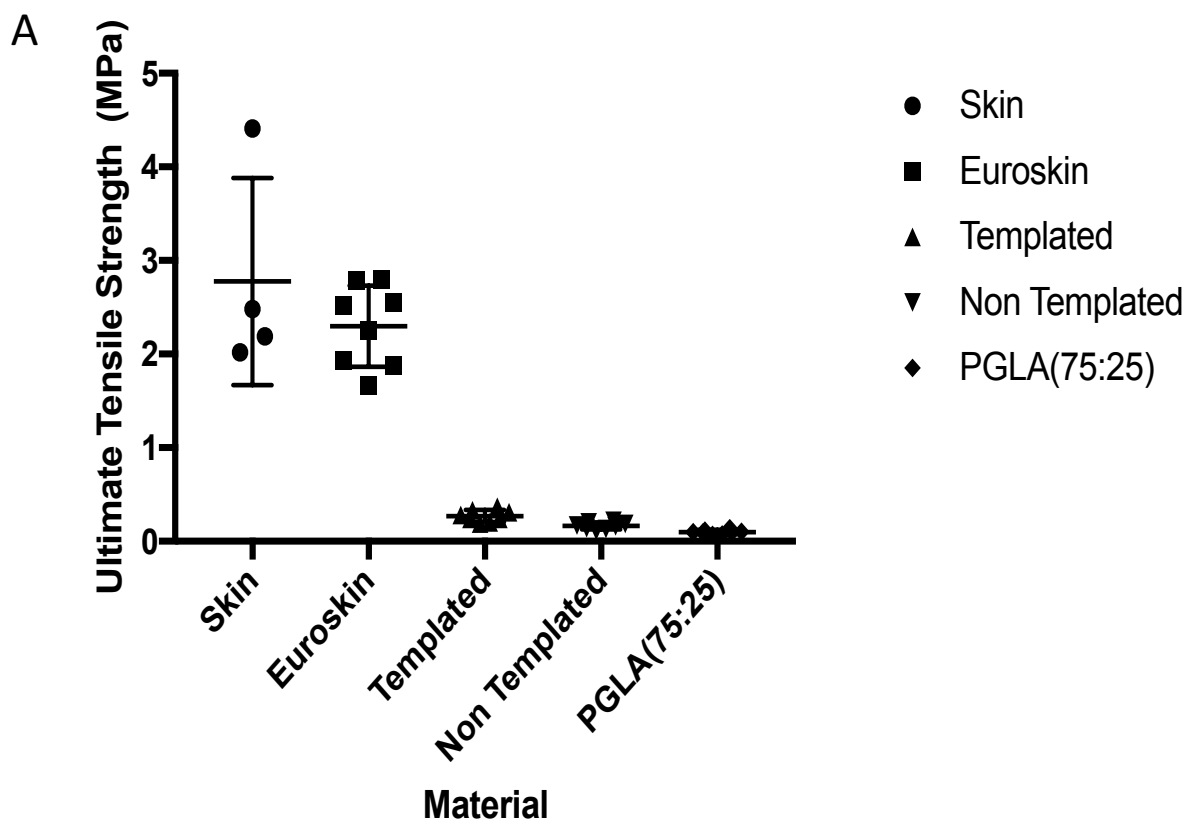
B
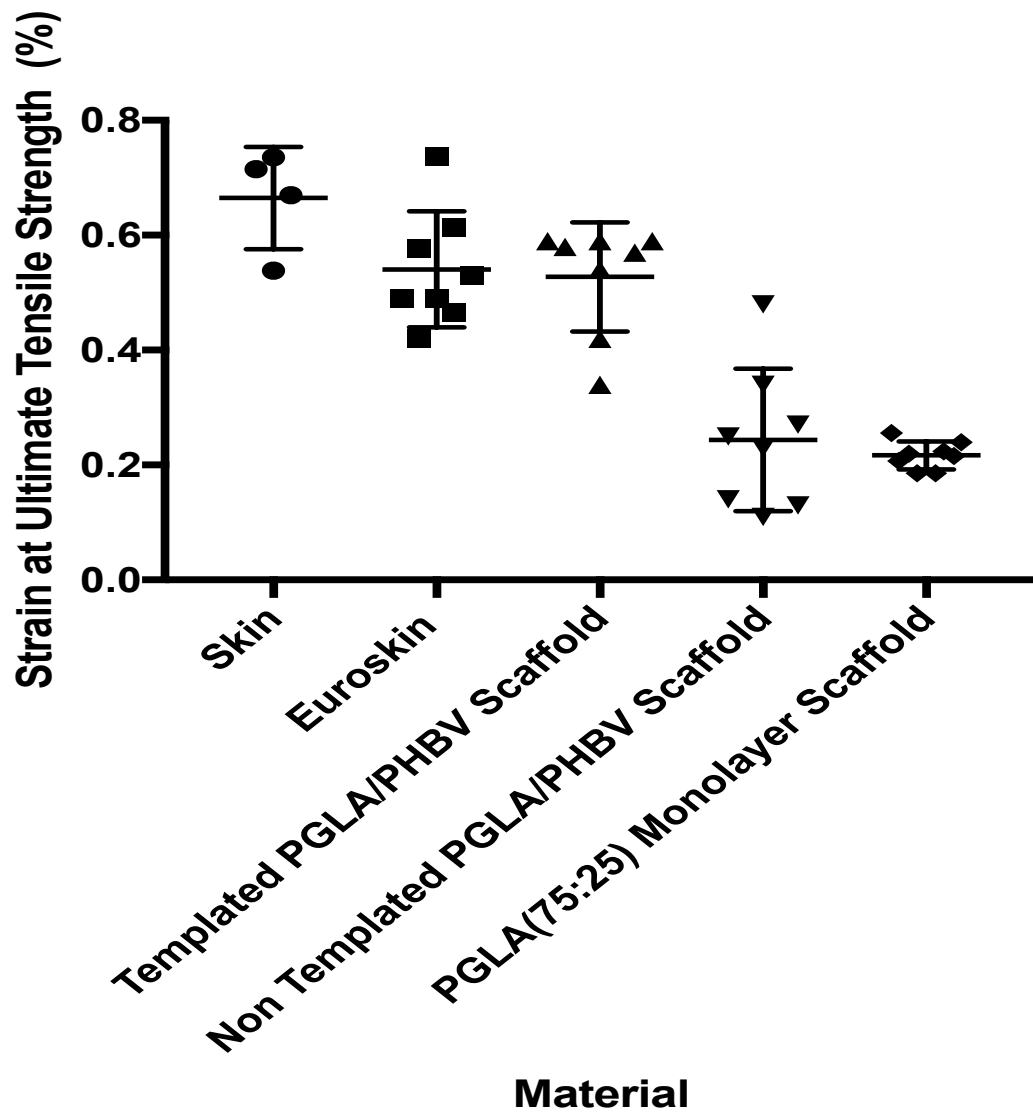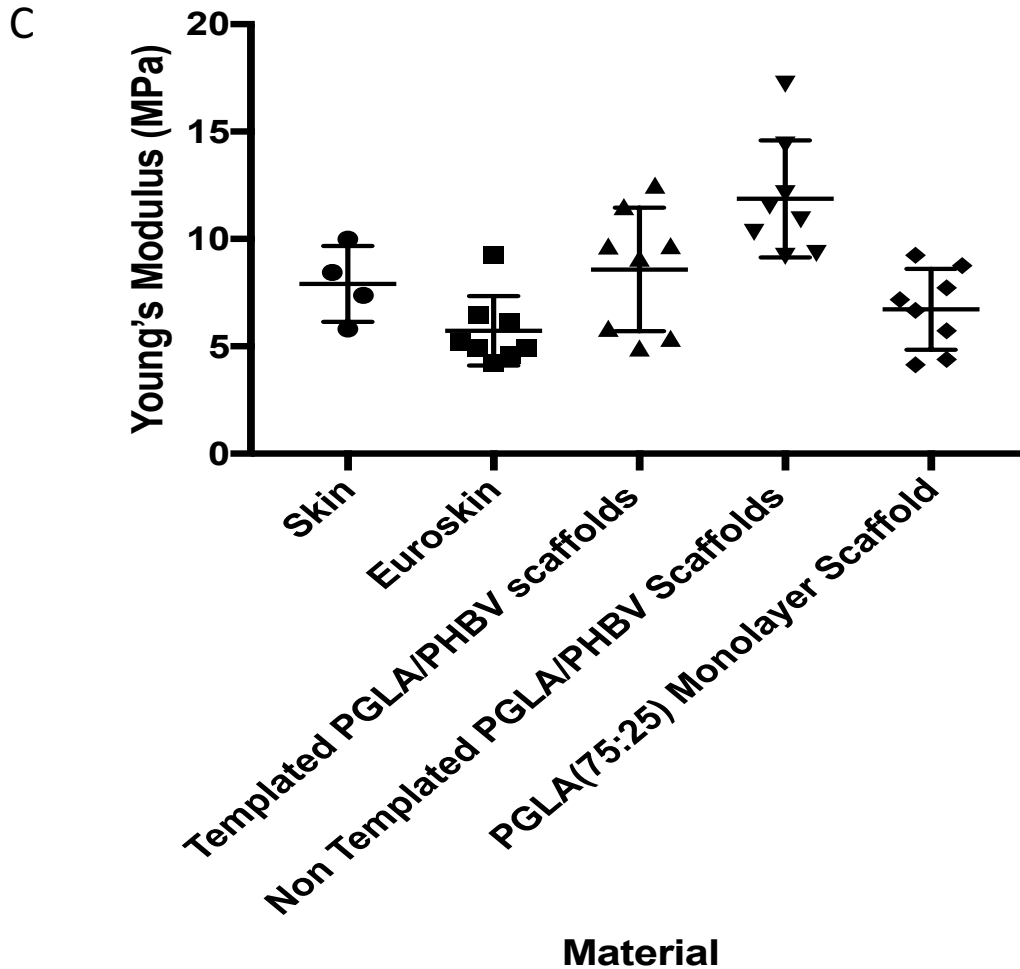
Figure 3.4: *Mechanical properties of human skin, Euroskin, templated trilayer PGLA (75:25)/PHBV, non-templated PGLA (75:25)/PHBV trilayer and PGLA (75:25) monolayer electrospun scaffolds. Mean±SEM (n=3). A=Ultimate tensile strength, B=Strain at Ultimate tensile strength and C=Young's Modulus.*

The non-templated trilayer scaffolds exhibited a significantly higher Young's Modulus compared to monolayer PGLA (75:25), skin and Euroskin, which highlighted its stiffness (Figure 3.4). Templated scaffolds had no significant differences in YM compared to skin and monolayer PGLA (75:25). Alternatively, the non-templated scaffolds had a significantly lower strain at ultimate tensile

strength compared to templated scaffolds, fresh skin and Euroskin but not significantly different to monolayer PGLA (75:25) scaffolds. The templated scaffolds had similar tensile properties to skin and Euroskin apart from Ultimate tensile strength, which is significantly higher in skin and Euroskin. However, when comparing templated scaffolds to non- templated and monolayer PGLA (75:25), the UTS was significantly higher in the templated samples. To conclude, adding the undulating features to the electrospun scaffold, resulted in material that was overall stronger and less stiff than non-templated scaffolds.

3.5 Evaluation of the stem cell niche in templated scaffolds

The middle layer of our electrospun scaffolds are templated, to mimic the undulations present in the interface between the dermis and epidermis. These templates are modelled on those scaffolds used for a similar application in corneal regeneration at the MacNeil laboratory based on a similar undulating pattern termed the Palisades of Vogt. Although there is published work on the dimensions of the rete ridges, it is challenging to exactly replicate this, as a result these templates were used in an attempt to assess the effect of proliferating cells in response to the undulations rather than having an exact replica. We used three metal templates (figure 6) all created in the same manner to produce the middle layer of PHBV for all electrospun scaffolds used in the experiments as shown in figure. The templates can be seen to consist of a regular honeycomb

pattern where the average diameter of the template feature was found to be 1.43mm (figure 7). A side view revealed the depth of the templates to be on average 566 µm (Figure 3.5).
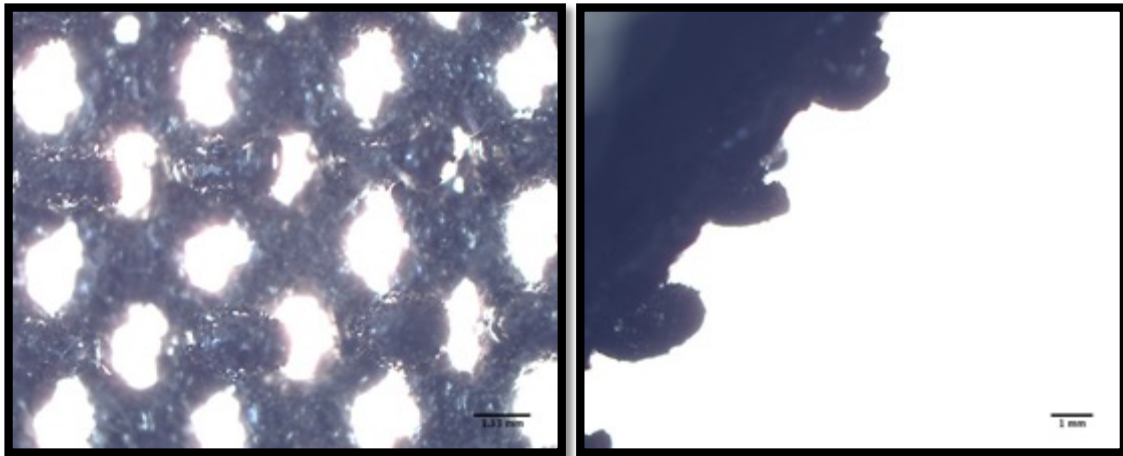
Figure 3.5: *Light micrograph of the templates in the stainless steel metal collectors illustrating the diameter and depth of the template.*
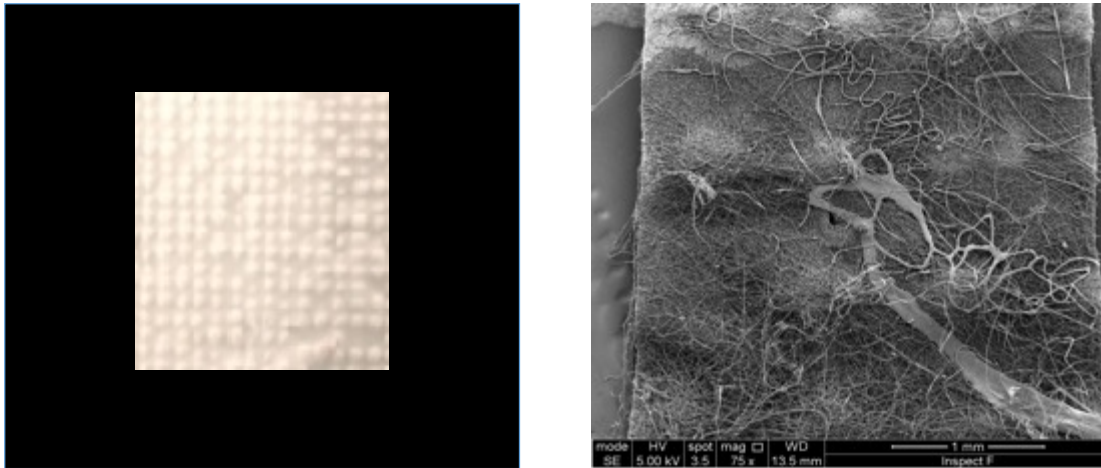
Figure 3.6 *Macroscopic and SEM images of the templated trilayer scaffold. These templates were translated into PHBV scaffolds where the undulating pattern was present both macroscopically and microscopically.*

3.6 Modification of Electrospun Scaffolds.

Vascularization of electrospun scaffolds can be viewed as one of the challenges to clinical translation. Inorganic polymers are attractive as they are inert and non-reactive with predictable breakdown products that are excreted in known physiological pathways. However, failure to develop and support a vascular system will result in failure of the material to integrate into the patient's own tissues. As a result, a lot of research has focussed on increasing the angiogenic properties of scaffolds. Table 3 summarizes some of the current published vascularization strategies for dermal scaffolds.

Strategy	Mechanism	Reference
Innate properties of the scaffold	Pore size and interconnectivity (180)	Allows space for infiltration of vessels of varying sizes
Treatment of scaffolds	Plasma treatment (182)	Proposed
Alternative polymers that are proangiogenic	Example: PolyN acetlyglucosamine NAG (183)	
Additives to scaffolds		
Molecular	Vascular Endothelial Growth Factor (184), (185)	Directly influences new and existing vessel growth by endothelial cell proliferation
	VEGF and Heparin (136)	Heparin allows for better binding of VEGF to enhance its effect
	Basic Fibroblast Growth factor (186)	Chemoattractant to fibroblasts – this releases

		factors that promote angio and vasculogenesis
	Platelet derived growth factor (184)	Stimulates proliferation of endothelial cells
	Alginate + Basic Fibroblast Growth Factor (187)	Chemoattractant to fibroblasts which secrete factors to stimulate blood vessel ingrowth
	Ascorbic Acid (110)	Co factor in collagen cross linking – thought to increase collagen which in turn increases the ingrowth of blood vessels into scaffolds
	Oestrodiol (112)	This is proangiogenic-thought to stimulate blood vessel ingrowth into scaffold.
	2 Deoxyribose (188)	As for oestrodiol, good alternative to VEGF

Cell Based	Human dermal microvascular endothelial cells (189)	Proangiogenic
	Umbilical vein endothelial cells (190)	Progangiogenic
	Endothelial progenitor cells (191)	Proangiogenic
	Mesenchymal stem cells from: Adipose tissue . (192) Bone marrow (193) Umbilical cord (194)	Proangiogenic, thought to differentiate into desired cell type depending on conditions.

Table 3: *Summary of current strategies to induce vascularity into scaffolds.*

As observed there have been many substances added to electrospun scaffolds to enhance their vasculogenesis. Several such approaches have been investigated in the MacNeil group. One such is the addition of estradiol to electrospun scaffolds. This was investigated by Mangir and colleagues where

they used PLA containing various concentrations of oestradiol and evaluated its angiogenic potential using a chick chorionic membrane assay(112). Their research revealed that these scaffolds increased ECM production and stimulated angiogenesis. The proposed use of this scaffold was for pelvic floor repair in females.

Possible issues with this would be the effect of systemic oestradiol absorption, although the dose of oestradiol used in the scaffold is likely to be very small, the clinical effects of this is yet unknown. Another strategy investigated adding ascorbic acid in two forms l-ascorbic acid (AA) and ascorbate-2-phopshate (A2P) to PLA scaffolds. This resulted in an increase in collagen production by fibroblasts(110). Although ascorbic acid is not a hormone and a vitamin, its proposed use as part of a scaffold is not what it is licensed for in humans i.e. topical and oral, as a result, this would also have to be approved before being allowed into the clinic. Other strategies include the addition of products that indirectly increases scaffold vascularity. For example, heparin when added to scaffolds stimulate the formation of new blood vessels. In order to use this property of heparin on scaffolds efficiently, chemicals like chitosan are added to the scaffold used to increase its heparin binding capacity [5150].

Our model did not include any additives hormonal or otherwise to stimulate neovascularization specifically as ultimately this would have to undergo rigorous

certification for human use. Addition of chemicals inorganic or organic alike, converts an electrospun scaffold from a medical device e.g. like biodegradable sutures or a metal plate for bony fixation or a joint replacement unit to a drug releasing device.

Any structure that contains or releases a chemical that can potentially react will contend with a significantly lengthy and challenging regulatory process, to get these to the clinic. As expected there will be the need to demonstrate via way of animal studies and then a clinical trial that these additives do not have any adverse effects on the physiology of the patient. Also there is a need to demonstrate that this is significantly better than the current materials that are being used to tackle the clinical problem in question.

Vascularization is essential for any wound to heal, however the skin has the ability to revascularize as a part of wound healing process given the right conditions (free of infection and the absence of excessive inflammation) are met. This is because signalling proteins are concentrated at the wound edges, which act as a strong drive for endothelial cells to migrate across the wound bed. We have therefore focused on the ultrastructure of the electrospun scaffolds and used only two polymers which are known to facilitate and support the migration of skin cells without any additives. This approach is based on the fact that the microstructure of skin is key to cellular organization and cell/cell

communication creating a homeostatic loop of continuous regeneration as and when needed. Once polymer provides the right scaffold for the patient's cells to proliferate and migrate, endothelial cells and vasculogenesis should occur.

In summary we have described in depth the polymers chosen for our scaffold and the reasoning behind seeking to provide the microstructure of a templated trilayer construct. We have outlined the various strategies that many research groups have published for optimizing tissue engineered dermal scaffolds. Furthermore, we have outlined the properties both essential and desirable of a dermal scaffold for clinical use and the strategies used to vascularize scaffolds. We have emphasized that for the purpose of this application to be used in medium sized (cannot directly close) full thickness defects, we aimed to keep the scaffolds free from chemical or hormonal additives which would convert the scaffold from being a medical device to a potential drug releasing device.

Chapter 4

Evaluation of how a novel method of spinning a basement membrane substitute will encourage epithelial stromal organization: Monolayer vs Trilayer Electrospun Scaffolds

4.1 Introduction

Basement membranes are thin sheets of extracellular proteins situated in close proximity to cells. They function to influence tissue compartmentalization and cellular phenotypes. The main constituents of all basement membranes are collagen IV and laminin. These are connected by nidogens (sulphated monomeric glycoproteins). Cells are connected to the basement membrane via glycoprotein integrin receptors. Collagen type IV is the most widespread

basement membrane component, its structure and arrangement confers mechanical stability (196)(197). Laminins (several heterotrimeric forms: laminin 1 to 7) also make up a significant proportion of the basement membrane. Proteoglycans are another important basement membrane constituent of which perlecan is the most abundant (198).

These proteins allow the membrane to adjust function at specific anatomical sites. For example, thin, continuous, extended sheets of basement membrane that maintain integrity and stability are needed over long distances in the vascular system. Additionally, sufficient mechanical stability is needed to resist the high shear forces at the dermal-epidermal junction in the skin. These proteins, by subtle phenotypical changes based on their anatomical locations are able to provide these properties to the basement membrane, the integrity of which is crucial for the survival of any organ system(199).

The extracellular matrix consists of structural and functional proteins arranged in a unique 3D structure and occupies the spaces between cells(167). The ECM of the basement membrane contains a mixture of extracellular proteins which include: laminin, collagen type IV and entactin(200).

Collagen type VII is a structural basement membrane component which forms fibrils, anchoring the basement membrane to the underlying dermis. Both keratinocytes and fibroblasts contribute to the formation of the basement

membrane in the skin(201). The basement membrane and the underlying dermis regulate keratinocyte growth and terminal differentiation, and thereby influence the structure of the overlying epidermis and its crucial barrier function(202). Therefore, it is desirable in organ regeneration to consider including a feature that can mimic or act like a basement membrane to further simulate the structure that is being engineered.

The use of electrospun scaffolds as a dermal regenerative template consisting of single polymers is an accepted technique (203). Simple monopolymer electrospun scaffolds provide a surface for cellular attachment and proliferation with eventual organization of cells and their products into dermis and epidermis. However, cells in the organ systems that we are trying to replicate exist in 3D structures within their respective tissues and environments. Culturing cells on a monolayer eg tissue culture plastic or a single polymer electrospun scaffold does not replicate this environment or provide a true potential for multi layered cell culture(204). Skin has two main layers separated by a specialized basement membrane. It would be desirable to create a scaffold which has the capacity to support different cells to proliferate and migrate and simultaneously communicate with each other in order to function as a unit.

We hypothesise that incorporating this into our scaffolds will encourage epithelial-stromal organization which is seen very clearly in native human skin.

In order to do this, we propose to use the electrospinning technique to combine two different polymers in three distinct layers thereby creating a trilayer electrospun scaffold. The PHBV or the basement membrane mimic will be sandwiched in between two layers of PGLA 75:25 which will support cellular growth (Figure 4.1).

PGLA(75:25)
PHBV
PGLA(75:25)
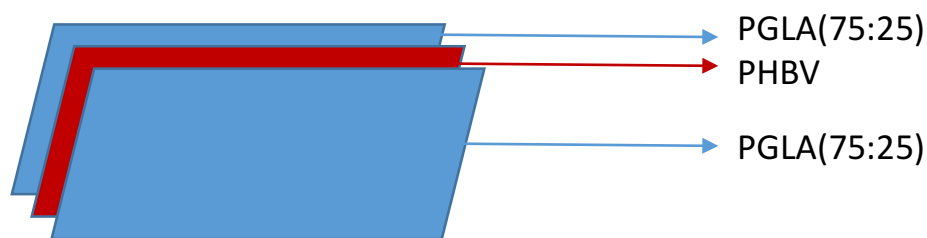
Figure 4.1: *Illustration of the trilayer electrospun scaffold, consisting of a middle layer of PHBV, in between two layers of PGLA 75:25.*

This method has been previously established in the MacNeil group using PLA and PHBV(205). A key feature of this technique when spinning two polymers at the same time is a method to prevent delamination of the different layers post spinning, which can occur given the different properties of the polymers. To do this we used 8mls of PHBV in 4 syringes (2mls each) to create the first layer on the template. The second layer was a hybrid consisting of 4mls of PHBV and 4 mls of PGLA 75:25, the third layer consisted of 8mls of PGLA 75:25. Once this was complete, the templates were removed and the scaffolds were flipped over to then add another hybrid of 4mls of PHBV and 4 mls of PGLA 75:25 on the

other side of the middle layer of PHBV, and finally 8 mls of PGLA 75:25 to create

the outer surface of the scaffold. <mark>This is illustrated below</mark>.
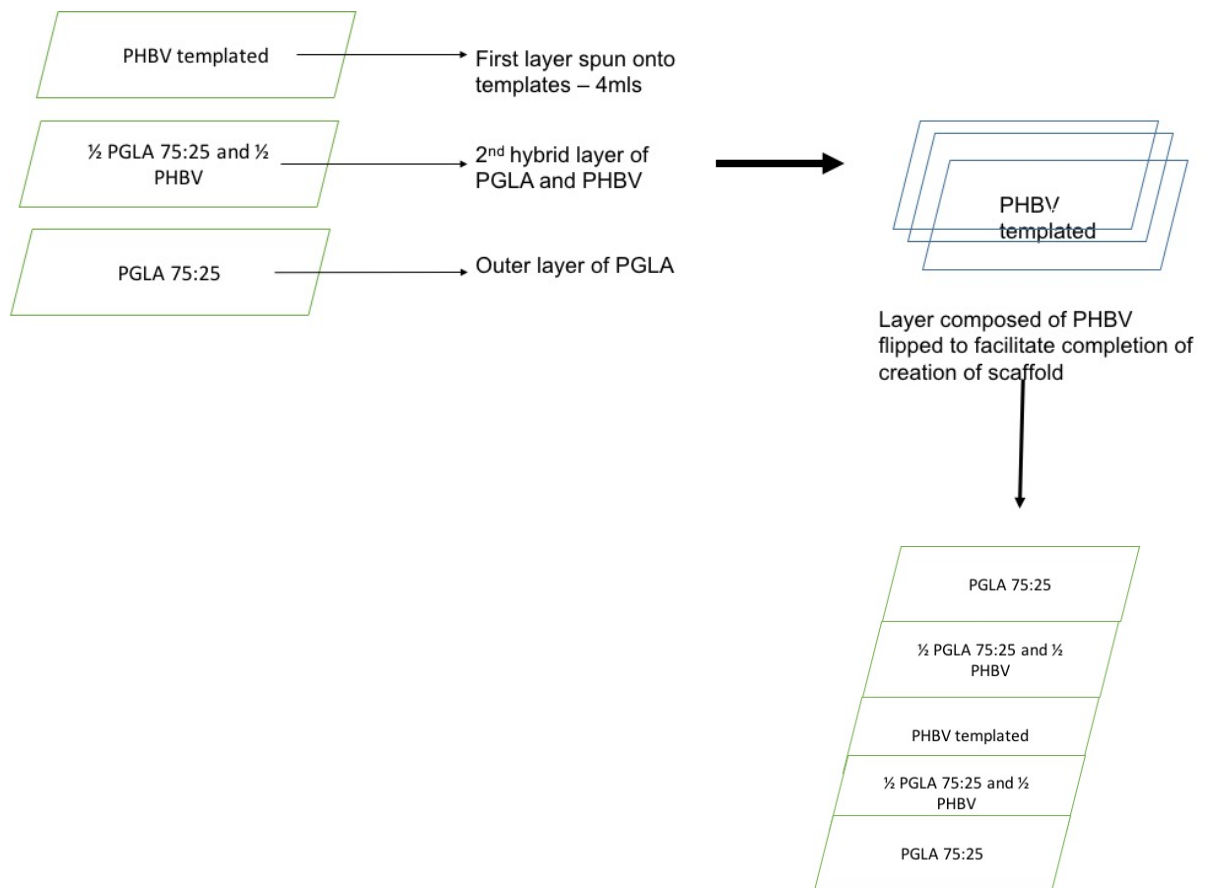
Figure 4.1b: *Cartoon to illustrate the process of spinning the polymers to prevent delamination during experimentation. The hybrid layer was essential where both polymers were mixed to facilitate this.*

In this chapter <mark>we aim to create electrospun scaffolds with a central layer of nano-porous and nanofibrous PHBV securely placed in between two layers of microporous and microfibrous PGLA (75:25).</mark> PHBV was chosen because it has

been shown to restrict cellular movement between two layers of different cells but still be porous enough to allow communication via chemical signalling between the two cell types(126). This is essential as the structure of the epidermis is dependent on the signalling factors produced by the cells of the epidermis and the dermis in addition to the underlying basement membrane.

PGLA 75:25 was chosen based on previous work that aimed at establishing a suitable polymer that would sustain the attachment and proliferation of fibroblasts and keratinocytes. Most importantly, it degrades with a decline in its mechanical strength at about five months post implantation which is an appropriate time match for wound healing and skin regeneration (124).

The experiments in this chapter compare the performance of a monolayer of PGLA 75:25 to a trilayer electrospun PHBV/PGLA 75:25 electrospun scaffolds in supporting the migration and proliferation of isolated human dermal keratinocytes and fibroblasts. We also aim to investigate the effect of the trilayer scaffolds if any, on the migration of skin cells from skin explants and their spatial organisation compared to monolayer scaffolds.

4.2 Quantitative analysis of activity of cultured cells on monolayer versus trilayer electrospun scaffolds.

4.2.1   Alamar Blue assay

Absorbances for cells and skin explant seeded on monolayer PGLA 75:25 and trilayer PHBV/PGLA 75:25 electrospun scaffolds were read. The absorbance for scaffolds of each type seeded without cells and explants were subtracted from these values for each time checkpoint at day 7, 14 and 21 (Figure 4.2.1). It was demonstrated that cellular constructs were viable throughout the 21-day testing period on both scaffolds types. Metabolic activity in the monolayer electrospun scaffold constructs progressively increased from day 7 to day 21. There were no significant differences in cellular metabolic activity when isolated cells were seeded on monolayer vs trilayer electrospun scaffolds apart from day 7 with the monolayer constructs having significantly higher metabolic activity versus trilayer at this time checkpoint. Although in absolute terms, the trilayer constructs had lower metabolic activity going forward to 21 days, this was not statistically significant. With regards to constructs with skin explants, it was found that there was significantly higher metabolic activity in those seeded on monolayer scaffolds compared to those seeded on trilayer scaffolds throughout the experimental period (Figure 4.2.2).
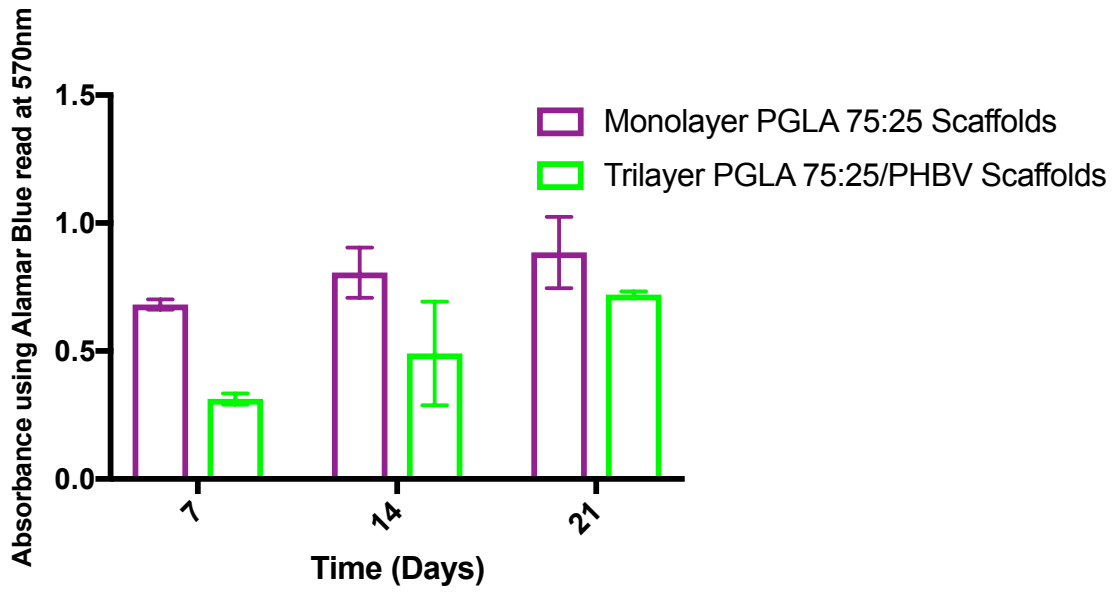
Figure 4.2.1 *Metabolic activity of isolated human dermal keratinocytes and fibroblasts on monolayer PGLA 75:25 electrospun scaffolds and non-templated trilayer (PGLA 75:25/PHBV) electrospun scaffolds over a 21-day experimental period of culture, as determined by absorbance of AlamarBlue stain (mean±SEM (n=3).*
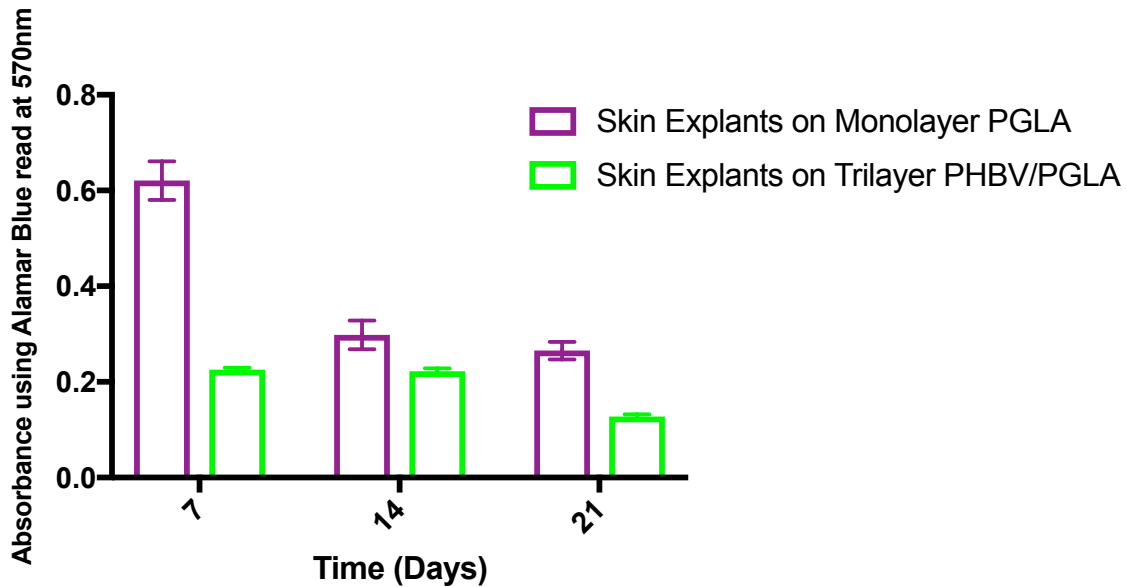
Figure 4.2.2 *Metabolic activity of skin explants on monolayer PGLA 75:25 electrospun scaffolds compared to non-templated PGLA 75:25/PHBV electrospun scaffolds over a 21-day experimental period of culture, as determined by absorbance of AlamarBlue stain (mean±SEM) (N=3).*

4.2.2 Sirius Red Assay

At day 7, 14, and 21 post seeding, Sirius red assays were undertaken to estimate the total collagen produced overtime by the isolated human dermal fibroblasts and keratinocytes seeded on the monolayer and trilayer electrospun scaffolds (Figure 4.2.3).

The aim was to quantify the ability of the electrospun scaffolds to support cellular proliferation and function by measuring the collagen production by

fibroblasts. We also wanted to determine if there were any differences between the monolayer PGLA 75:25 and trilayer PHBV/PGLA (75:25) constructs.

With regards to the cellular constructs, collagen production increased in the monolayer scaffolds over the 21-day experimental period and was significantly greater compared to collagen production in the trilayer constructs only on days 14 and 21.
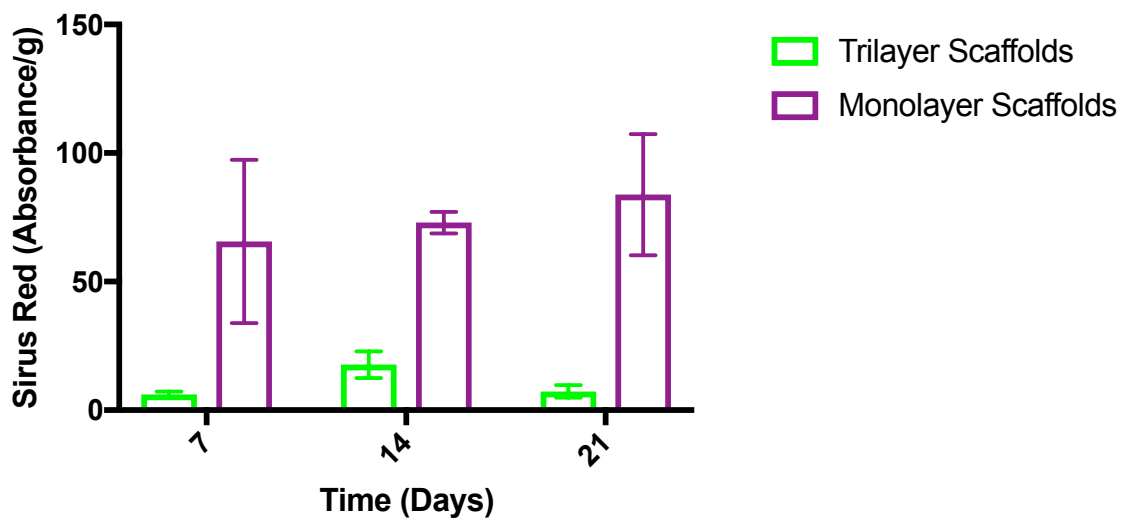
Figure 4.2.3 *Sirius red staining after 7,14 and 21 days of cultured isolated human fibroblasts and keratinocytes on monolayer and trilayer electrospun scaffolds. (N=3±SEM).*

4.3 Qualitative evidence of cellular migration within monolayer PGLA 75:25 electrospun scaffolds.

4.3.1 DAPI Staining

This was undertaken for both cell and skin explant constructs fixed at day 7, 14, and 21 to demonstrate cellular attachment and migration along fibres of monolayer PGLA 75:25 and trilayer PHBV/PGLA 75:25 electrospun scaffolds. Both layers were imaged such that the top surface is shown in the pictures for the trilayers to see the migration of keratinocytes, for the monolayers the orientation made no difference. We also wanted to detect whether there were any differences observed between the two. Good cell attachment and migration were demonstrated along the fibres on both monolayer PGLA 75:25 and trilayer electrospun scaffolds, which were comparable to each other in pattern and distribution (Figure 4.3.1) when isolated cells were seeded. However, when comparing constructs with skin explants , cells were seen to be migrating from the cut edge of the skin explants onto the surface of the monolayer scaffolds most predominantly at day 21 compared to trilayer constructs, which had the best evidence of cellular migration at day 14.
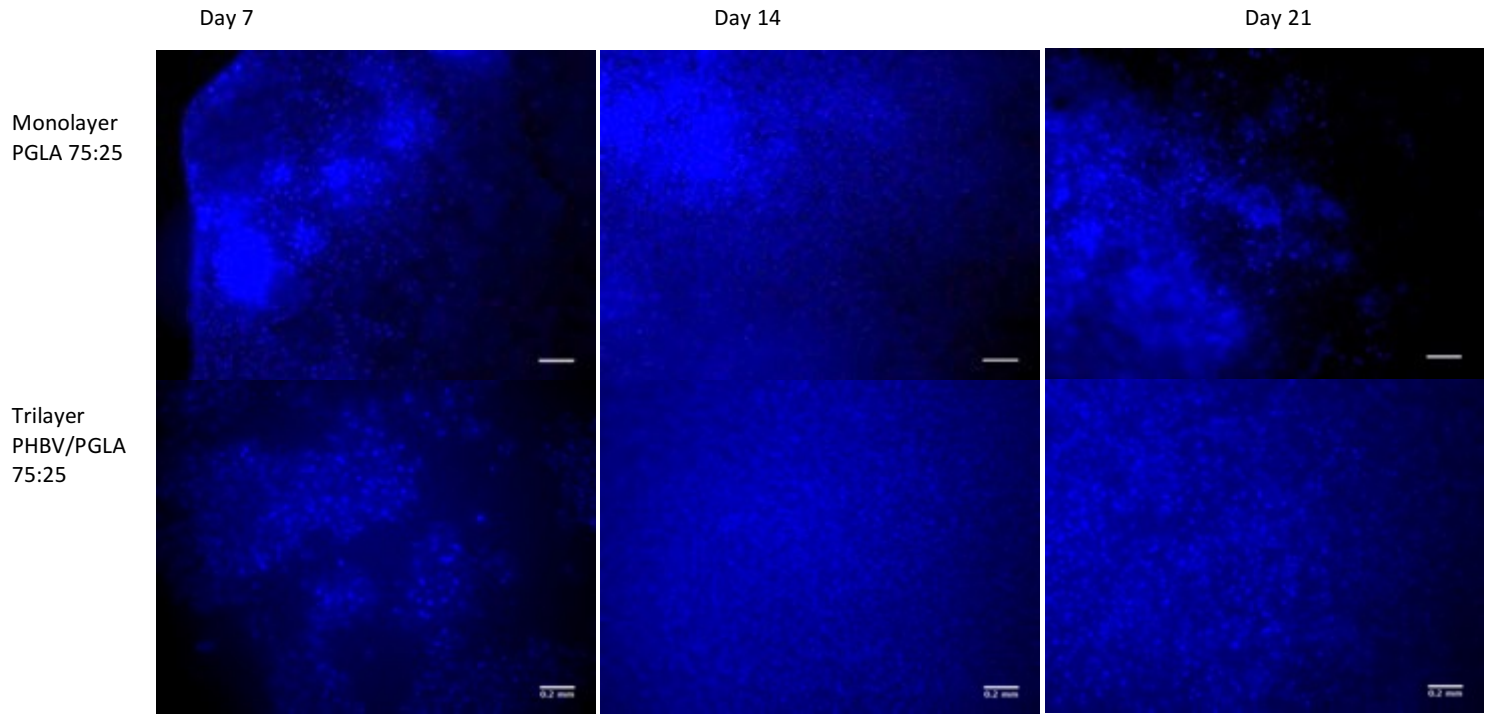
Figure 4.3.1 *DAPI staining of constructs (monolayer scaffolds and trilayer scaffolds) cultured with isolated human fibroblasts and keratinocytes. Scale bar =0.2mm.*

| Day 7 | Day 14 | Day 21 |

Monolayer PGLA 75:25

Trilayer PHBV/PGLA 75:25
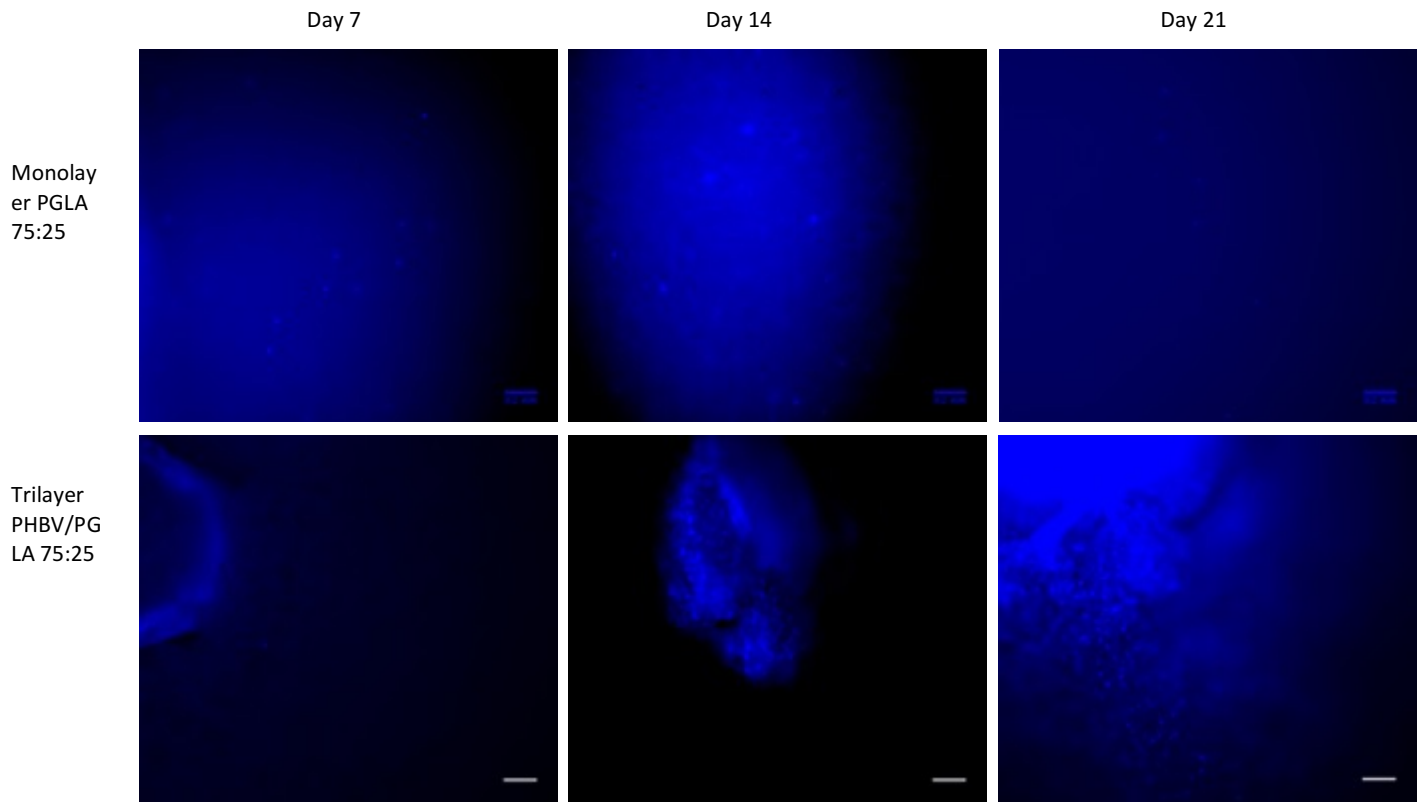
Figure 4.3.2 DAPI staining of constructs (monolayer scaffolds and trilayer scaffolds) cultured with skin explants. Scale bar =0.2mm.

4.3.2 Haematoxylin and Eosin Staining

For these experiments, confluent dermal fibroblasts were seeded on both Monolayer 75:25 PGLA and non- templated trilayer PGLA 75:25/PHBV electrospun scaffolds respectively and cultured for 48 hours followed by confluent isolated keratinocytes. These experiments were repeated using skin explants instead of isolated cells and fixed at days 7, 14, and day 21. Specimens were cryosectioned and stained with H&E according to the protocol outlined in the methods. The aim here was to obtain a quantitative assessment by way of

images to determine if there was any difference in the migration and integration of cells versus skin explants on both monolayer and trilayer scaffolds.. The cellular nuclei appear as blue/black and the cytoplasm and surrounding connective tissue appears pink. H&E stained constructs containing cell seeded monolayer and trilayer electrospun scaffolds demonstrated confluent cell populations on both scaffolds(Figure 4.3.4). A distinct difference was seen in the architectures whereby the trilayer scaffolds had a defined layer consisting predominantly of keratinocytes. The PHBV basement membrane layer is also visible in these constructs which is largely devoid of cellular material as intended. The fibroblast seeded layer is also seen in the H&E sections of the trilayer constructs. Compared to the monolayer scaffolds, which consist of both cell types in one polymer layer (Figure 4.3.3), the trilayer construct has a more similar architecture to that of human skin (Figure 4.3.4 and Figure 4.3.5). H&E sections of monolayer and trilayer electrospun scaffolds with explants demonstrate that there was less cellular migration from the cut edge of the explants and thereby less cellular proliferation along the fibres of the scaffold compared to using isolated cells (Figure 4.3.6).
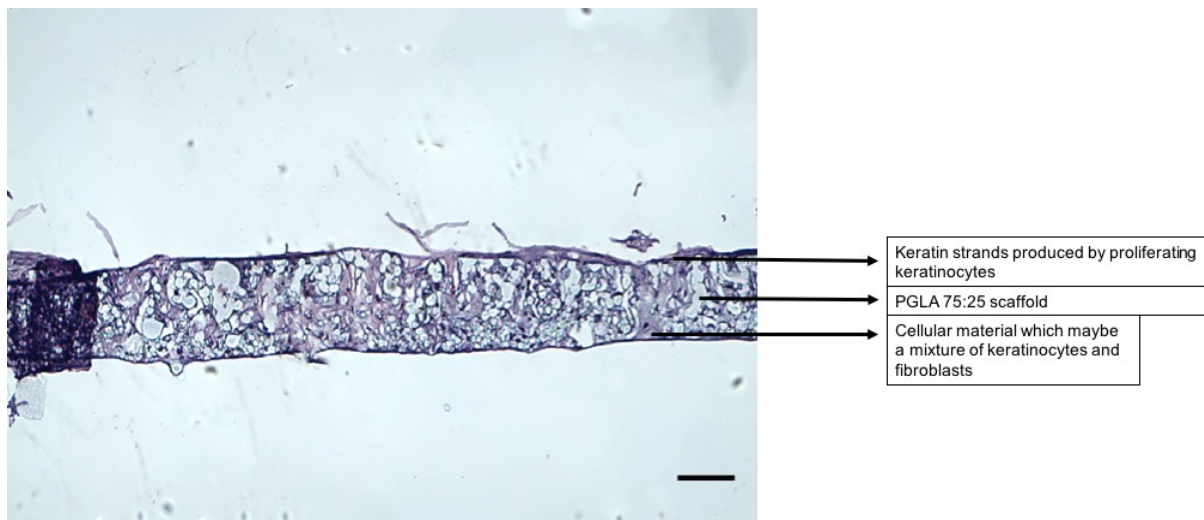
Figure 4.3.3. *Annotated light microscopy H&E stained section of isolated human dermal fibroblasts and keratinocytes cultured on monolayer scaffolds. Scale bar of 0.2 mm (10X magnification).*
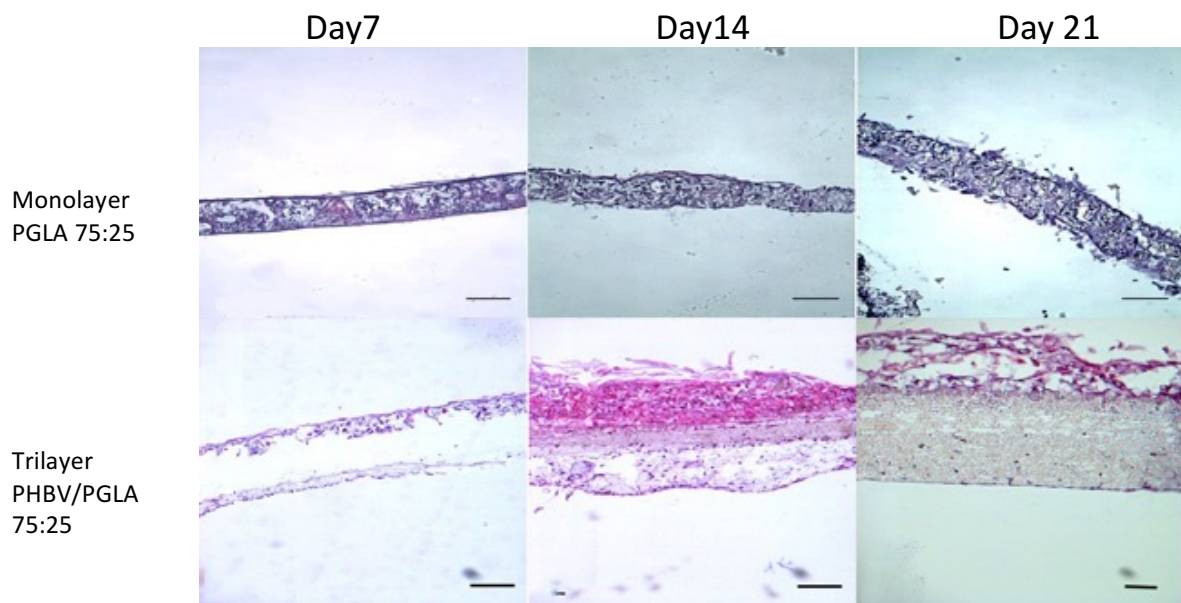
Figure 4.3.4 *Representative light microscopy H&E stained sections of isolated human dermal fibroblasts and keratinocytes cultured on monolayer and trilayer electrospun scaffolds. Scale bars of 0.2 mm (10X magnification).*

Monolayer scaffold showing generalized staining with no discernable cells or cellular material

Epidermis

Dermis

Neo epidermis cultured with isolated keratinocytes

Neo Dermis cultured with isolated fibroblasts

Epidermis

Dermis
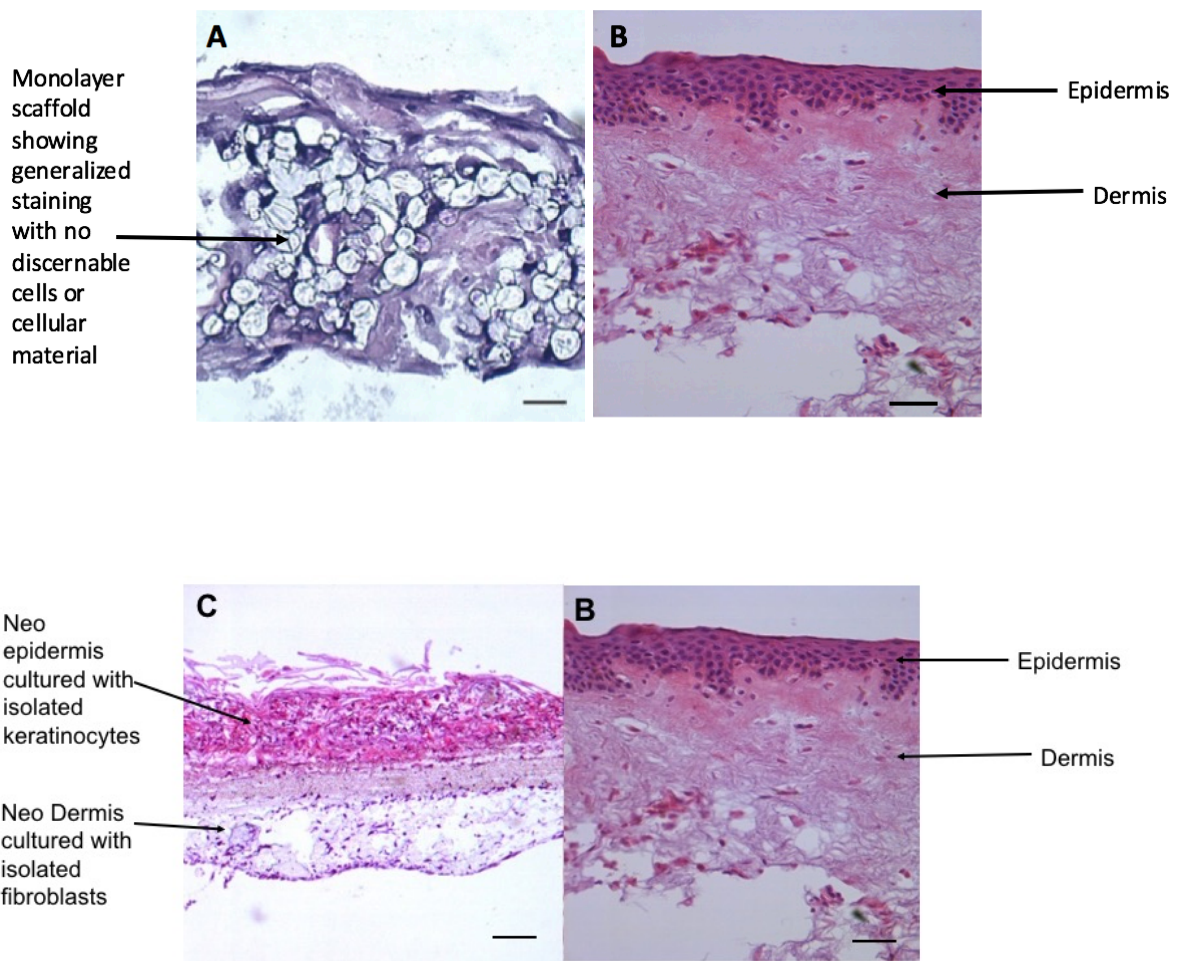
Figure 4.3.5 *Representative light microscopy H&E stained sections of isolated human dermal fibroblasts and keratinocytes cultured for 14 days on monolayer (x200)(A) and trilayer electrospun scaffolds (x40)(C) compared to human skin(x100) (B. These figures illustrates the definable layers of epidermis and dermis created by the trilayer electrospun scaffold technique when cultured with isolated fibroblasts and keratinocytes.*

Day 7
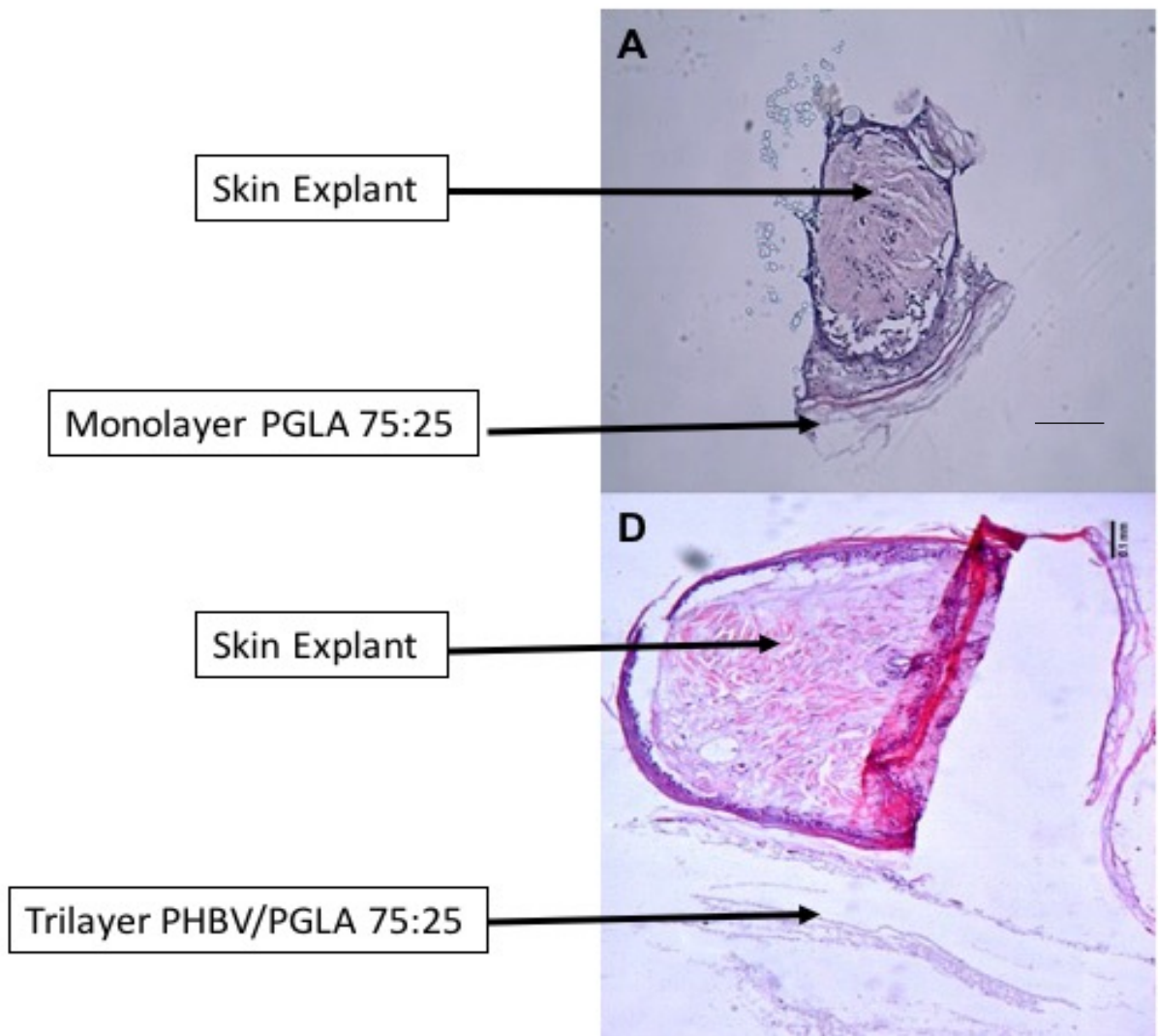
Figure 4.3.6 Representative light microscopy H&E stained sections of skin explants cultured on monolayer PGLA 75:25 (top) and non-templated PGLA 75:25/PHBV trilayer electrospun scaffolds (bottom), illustrating poor contact of skin explants on scaffold at day .. Scale bars of 0.2 mm (100X magnification).

4.4 Scanning electron microscopy

These were undertaken to demonstrate cellular migration and proliferation along the fibres of both isolated fibroblasts and keratinocytes and skin explant seeded monolayer PGLA 75:25 scaffolds respectively after 14 days in culture. We chose representative images at this time point as we would expect some cellular migration and proliferation from the edge of the skin explant onto the scaffold by 14 days. These images clearly illustrate cellular infiltration and proliferation on the fibres of the electrospun scaffolds (Figure 4.4.1). Furthermore, the production of extracellular proteins and collagen fibrils that have been deposited by the proliferating fibroblasts are visible on the surface of the scaffolds (Figure 4.4.2). Imaging was also undertaken for the skin explant seeded constructs to understand if there was any cellular migration at this time point. The image (4.4.3) shows very little in the way of cellular migration and proliferation in these constructs especially in the area surrounding the skin explants.
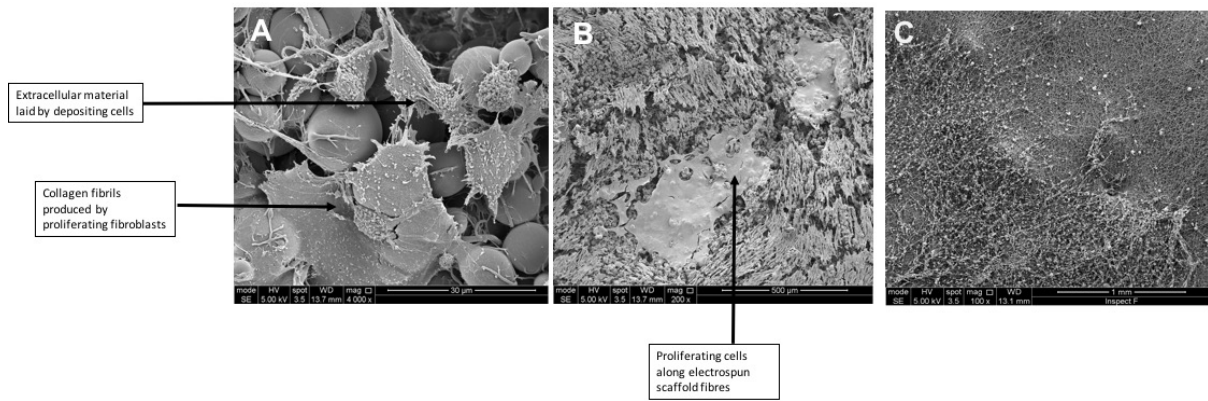
Figure 4.4.1 *SEM images of isolated human dermal fibroblasts and keratinocytes cultured on monolayer electrospun scaffolds (x4000 and x200 Magnification (A and B). C shows monolayer PGLA 75:25 scaffolds without seeded cells for comparison (x100 magnification)).*
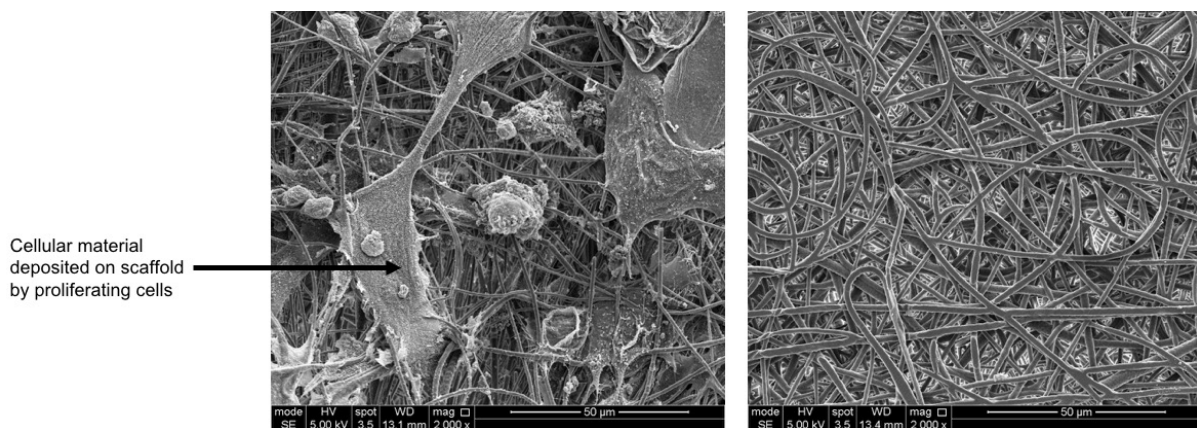
Figure 4.4.2 *SEM image of isolated fibroblasts and keratinocytes cultured on trilayer PGLA 75:25/ PHBV electrospun scaffolds at day 7(left). The figure shows deposition of extracellular material on the scaffold by proliferating cells. (x2000 magnification). The figure on the right is an SEM image of the same magnification of the non-templated trilayer scaffolds without cells for comparison.*

Skin explant on
monolayer PGLA
75:25 scaffold

Monolayer PGLA
75:25
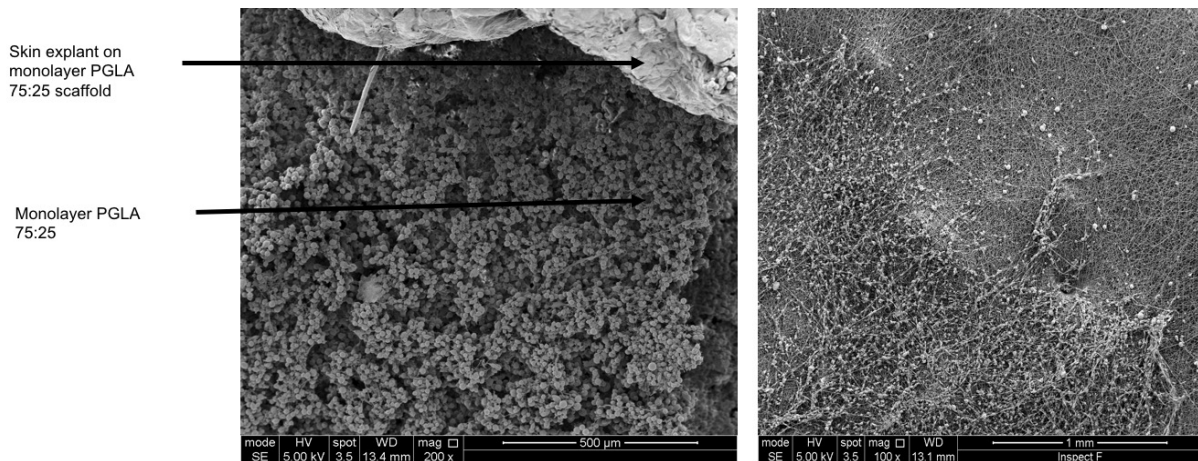
Figure 4.4.3 *SEM image of skin explants cultured on monolayer (PGLA 75:25) electrospun scaffolds(left) (x200 Magnification). SEM image of plain monolayer 75:25 electrospun scaffolds without cultured skin explant for comparison (right) (x100 magnification)*

4.5 Collagen IV Immunohistochemistry

This was undertaken on day 14 for monolayer and trilayer constructs seeded with isolated fibroblasts and keratinocytes. The aim of this was to determine if there was any organization of the collagen into a defined layer close to the PHBV polymer middle layer which acted as a nanofibrous/porous structure similar to that of the ECM of the basement membrane. Figure 4.5.1 demonstrates that in the monolayer constructs collagen is distributed randomly as deposited by the fibroblasts. However, in the trilayer constructs there is a distinct organization of the collagen confined to the layer that contained the fibroblasts and just adjacent to the PHBV layer. A comparison of collagen IV immunohistochemistry

of stained human skin made with H&E stained human skin is demonstrated in

Figure 4.5.2 to orientate the epidermis and the dermis.
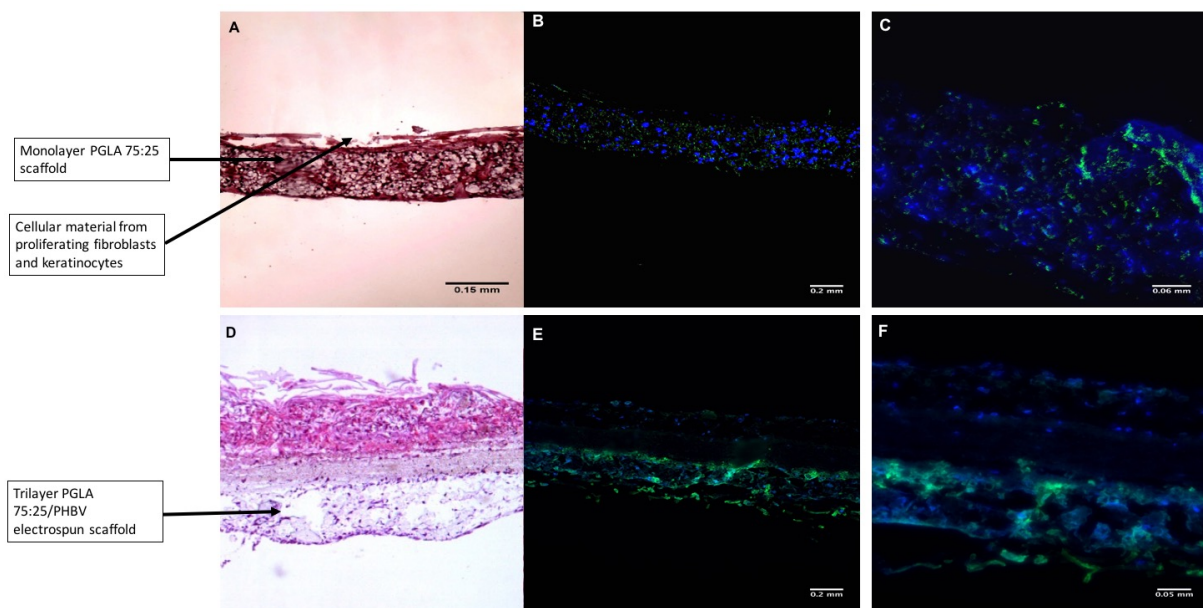
Figure 4.5.1 *Immunohistochemistry staining of collagen IV (seen as green) produced by cultured isolated dermal fibroblasts and keratinocytes comparing the collagen organization on monolayer PGLA 75:25 (B and C) and trilayer PGLA 75:25/PHBV electrospun scaffolds(E and F). Scale bars = 0.2mm (x100). H&E staining of the same constructs (A and D) are inserted for orientation.*
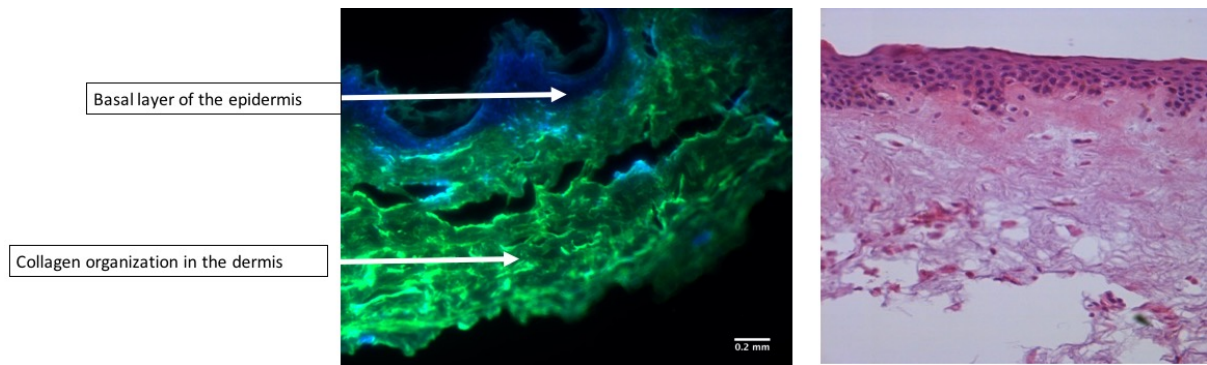
Figure 4.5.2 *Immunohistochemistry for collagen IV on human skin demonstrating the collagen organization in the dermis (left). H&E stained slide of human skin(right) placed as a reference to orientate the image on the left. Scale bars of 0.2mm (x10).*

4.6 Discussion

The experiments in this chapter aim to evaluate a novel method of spinning a basement membrane substitute to promote epithelial/stromal organization. In order to do this, we fabricated electrospun scaffolds consisting of two different polymers in three distinct layers. The two layers of PGLA 75:25 are cell supportive encouraging the growth of skin cells and the PHBV nanofibrous layer is meant to act like a basement membrane to aid definition of the neo epidermis and neo dermis.

It is known that PGLA 75:25 supports cellular growth and proliferation(124). Its microporous characteristics facilitate cellular movement through the scaffold and its microfibrous nature facilitates cellular proliferation, and ECM protein

and collagen deposition due to its high surface area. Constructs containing monolayers of PGLA 75:25 only therefore exhibited superior ability to support both isolated skin cells and skin explants in this regard when compared with trilayer constructs. This is supported by AlamarBlue and Sirius Red analyses as demonstrated above. This could be explained by the fact that the nanofibrous layer of PHBV in the centre of the trilayer scaffolds is impermeable to cellular movement compared to the PGLA 75:25 on either side. This layer is almost devoid of cells as a result , it serves to create a barrier between the neo dermis consisting mainly of collagen and a neo epidermis consisting of keratinocytes and keratin. However the middle PHBV layer is still porous enough for chemical signals from the fibroblasts to reach the keratinocytes in order to stimulate them to migrate, proliferate, and differentiate. This process takes time, whereas constructs with monolayer scaffolds, having all the cells mixed together will not have this lag as the cells are next to each other in the same area. As a result, the quantitative analyses reflect this, with significantly higher metabolic activity and collagen production in these monolayer constructs. This is the same for scaffolds seeded with skin explants. Furthermore, the DAPI images at 21 days of constructs containing skin explants seeded on monolayers, show significant cellular migration from the skin explants onto the electrospun scaffolds

comparable to that of isolated cells seeded on scaffolds. These results therefore support the use of PGLA 75:25 as one of the polymers in the trilayer constructs. However, when analysing the actual structure of the composites at day 14, it is clearly demonstrated that this alternative method of spinning a basement membrane into the scaffold defines the layers of the skin to a greater extent compared to monolayer constructs. Collagen deposition is more organized into a distinct layer with keratin, made by proliferating keratinocytes is also found in the opposite layer. As a result, the cellular organization of these trilayer constructs are more similar to that of human skin as illustrated in Figure 4.7.2 compared to their monolayer counterparts.

Another difference between using these scaffolds, which is very relevant to clinical translation, is its handling ability when undertaking the experiments.

The PGLA 75:25 monolayer scaffolds were very adherent to the foil onto which they were electrospun. They required prolonged soaking in PBS to release the scaffolds from the foil. Although this can be tackled by soaking in PBS before being used in the lab a better solution is most definitely required in order to upscale up for future clinical use. `Furthermore despite using the same volume of polymer in both the trilayer and monolayer scaffolds, the monolayers were very thin and did not support the skin explants well. They were almost transparent and tore very easily when handled.

We have demonstrated that it is possible to spin a basement membrane like layer into an electrospun construct and use a combination of polymers to simulate the various layers of the skin. This work is only part of the emerging techniques of using multiple polymers as part of the same scaffold construct to provide more definable anatomical and functional layers. Other combinations using this principle of polymer blending have been used for tissue engineered skin. For example poly (caprolactone) (PCL) was blended with collagen to improve its mechanical properties for tissue engineered dermis(206). Additionally in vascular tissue engineering, synthetic polymers like PDS are blended with organic proteins like gelatin and elastin to create the appropriate fibrous and porous network that will support endothelial cell development (207).

In summary we have created a trilayer electrospun scaffold consisting of a basement membrane like layer that has the ability to support organization of cells and extracellular matrix which appear more like native human skin compared to monolayer PGLA 75:25 scaffolds. The current results based on laboratory expanded cells suggest this structure has some benefit to offer with respect to spatial organisation of these cells.

There was not however any benefit to be seen to outgrowth of cells from tissue explants in this in vitro model.

Chapter 5

Evaluating split thickness skin interaction with dermal replacement scaffold

5.1 Introduction

In developing any tissue engineered skin substitute attention must be paid to the cell source that is going to be used to populate the scaffold. In this regard, both scaffold and cell source need to be synergistic in order to have an end product that is analogous histologically and physiologically to human skin. Skin replacement can be thought of as epidermal, dermal and composite (both epidermis and dermis present). When creating a dermal equivalent to be placed on a full thickness wound bed, a clear mechanism of epithelial cell source and attachment must be identified. For example, the commonly used Integra, requires a very thin split thickness skin graft (5-6/1000 inches) to be placed on it, either at the time of surgery (one stage Integra) or 2-3 weeks later (traditional Integra) which is attached to the Integra using skin staples, sutures or glue. In fact, all clinically available dermal replacements will require an epithelial cell source usually from the patient themselves. This can be challenging if the patient has no available sites. In this case a small sample of their skin can be taken to the lab where the epithelial cells can be isolated and cultured in sheets which are then transferred onto the prepared dermal wound bed. However, the use of isolated cells to populate a bioengineered dermis has been fraught with challenges and as a result its clinical uptake has remained low despite this being a breakthrough technique since the 1980s(1). The most documented used

approach for isolated skin cells clinically is CEA(208)(100). The principle of using CEA is as follows: these are integrated sheets of cultured keratinocytes that are grown in clean room facilitates from the patient's skin as a cell source. Once the biopsy is taken, cells are isolated and cultured until confluent sheets of cells are formed to be transferred back to the patient onto an appropriately vascularized wound bed. This wound bed should ideally have a dermal matrix -which may be the patient's own dermis (mixed depth burn where the burn has been excised to mid dermis depth), cadaveric dermis, or an artificial dermal template. The process in itself of producing CEA is successful it is a long standing, tried and tested technique of cell culture. However, there are significant barriers that have resulted in a low clinical uptake. Firstly, its production has to be cost effective. Although cell culture itself is affordable, for human use, there needs to be clean room facilitates and trained staff in order for this to be approved for human use by local ethics committees. There are very few laboratories that can therefore accommodate this not just with respect to facilitates and personnel but also having the appropriate HTA licensing which allows this work to be carried out in the facility. Not only does this reflect the increased cost of using such products but also transport and delivery costs is considered when departments decide on a product for clinical use especially if they need to be sourced nationally. Furthermore, the timing of delivery of cells need to be exact,

once cells reach confluence if there are not used within 2-3 days they will not attach to a wound bed. As a result, the product in itself does not meet the flexibility that is demanded with a product that is suitable for routine use clinically, ideally it should be off the shelf. This is aptly demonstrated by Hernon et a, where they reported in a 10-year audit of CEA use in burns, nearly 50% of the sheets were wasted between CEA production and delivery to patients(78). Flexibility in a product is one of the key features that contribute to routine clinical uptake and explains why dermal regenerative templates like integra and Matriderm have enjoyed long standing clinical success as they do not require cultured cells but very thin epithelial autografts. Furthermore, tissue engineered products are often only used in the context of larger areas of skin resurfacing, be it burns, or large skin lesions when there are not enough autologous donor sites to safely resurface. For acute burns by and large these patients are physiologically unstable and their suitability for operative intervention are determined by daily clinical reviews. As a result, any change in the patient's physiology may offset surgical intervention which may result in wastage of the product. In addition the outcome of using CEA has been described as inconsistent in published case series throughout the years.(209) Take is a visual objective outcome measure used to determine if application of a skin substitute or a graft has been successful. It is mainly denoted by adhesion to the wound

bed and a lack of infection. The take of CEA sheets is inconsistent and this actually is reflective of any large burn as several factors influence take, namely the vascularity of the wound bed, and the extent of the immunological hit the patient has had. These factors are not easily influenced or predictable by objective measures (e.g. a blood test) therefore using CEA sheets may well have not have been inferior to split skin grafts however in order for a product to assume routine clinical use, it has to be seen as superior to the current gold standard, which in the case of skin resurfacing is an autologous skin graft. The bias here lies in the fact that there are few robust measures in assessment of autologous skin grafts or skin substitute use. Most published work consists of cases series and expert opinions which actually maybe the highest level of evidence that one may obtain in this field of reconstructive surgery. Successful outcome is also affected by the experience of the team treating the patient in firstly making the most appropriate judgement of the material to use, timing and technique application. As a result, it is not surprising that some groups who may have more clinical experience and access to close laboratory facilities to produce CEA sheets have reported better than average results in their experience with CEA(208). In addition, these sheets are fragile, and not easy to handle. This technical feature is a minor deterrent as there have been carrier mechanisms that have developed to successfully overcome this(78). Therefore,

one can consider the need to steer away from laboratory expanded cells to populate scaffolds and use an alternate cell source with respect to developing a tissue engineered biodegradable substitute. Ideally this should come from the patient themselves which reduces the immunogenicity associated with the process and thereby increase the chances of successful take. Our proposal therefore used the concept of Meek Grafting as a source of cells to populate the scaffold. The principle of wound healing here is the same as a meshed graft, in that epithelial cells undergo conformational change, migrate to the wound edge until they meet other epithelial cells and then recreate epithelial connections. This principle is also used in the technique of fibroblast culture from skin explants. Here instead of using an enzymatic process, a skin sample is taken as soon as it is harvested, it is minced into fine pieces and placed into 6 well plates containing 10% DMEM. Attachment and outgrowth of keratinocytes occur at day 3 and fibroblasts at day 7(31). This is advantageous in that in cases with small amount of tissue, it is a more reproducible technique, it is technically simpler and avoids over digestion, reduction of cell viability, and subsequent attachment of cells when not handled properly. Therefore, the principle of using skin explants as a cell source to populate the trilayer templated electrospun scaffolds in principle should result in skin cells leaving the cut edge of the skin explants, and migrating along the fibres of the electrospun scaffold.

This concept of explant use as a cell source has been established in the MacNeil laboratory and translated into clinical practice in the area of corneal regeneration. In brief, damaged corneas are regenerated by excising the non-viable tissue, and using an amniotic membrane scaffold with small pieces of limbal explants from the contralateral normal eye. The explants are held in place with fibrin glue. At 1.5 years, 76% of patients had an epithelized, stable corneal surface(132). Therefore, the aim of the experiments in this chapter is to assess the ability for skin explants to be used as a cell source to populate the trilayer, templated PGLA (75:25), and PHBV electrospun scaffolds to create a composite skin substitute. To do this, we needed to establish a control group with a standard to which we would be aiming for with regards to cellular proliferation and migration. The first set of experiments establishes the ability of the trilayer electrospun scaffolds to support isolated human dermal fibroblasts and keratinocytes. To do this, we used as a control, the original tissue engineered skin, consisting of a dermal scaffold of DED, to which we populated fluorescently labelled isolated human dermal fibroblasts and keratinocytes on either side. Additionally, we seeded the same cells on the trilayer electrospun scaffolds using the same technique, and raised all constructs to an air liquid interface after 3 days. They were cultured for a further 7, 14, and 21 days respectively. Previous work conducted in the MacNeil lab has already established the ability of

electrospun scaffolds using various polymers to support the migration and proliferation of isolated human dermal fibroblasts and keratinocytes(124)(205). However, it was important to establish, as a control the ability for the templated trilayer scaffolds to support epithelial cells. Once this was completed we then used skin explants laid onto the scaffolds as a cell source. They were cultured for 3 days with Green's media and raised to ALI, continuing for another 7,14, and 21 days. The data therefore represents a comparison using both qualitative and quantitative methods to determine the ability for skin explants to be used as a cell source to populate the electrospun scaffolds.

5.2 Culture of isolated keratinocytes and fibroblasts on templated versus scaffolds compared to tissue engineered composites using DED

CellTracker Fluorescence Staining

This was undertaken at 48 hours following seeding of both isolated human dermal fibroblasts and keratinocytes on electrospun scaffolds and DED to confirm their presence. Fibroblasts were used between passage 4-9 and keratinocytes between 1-3. The images seen demonstrate green fibroblasts and red keratinocytes were successfully seeded on scaffolds before raising it an air liquid interface and also the differing architectures of the scaffolds used in experiments (Figure 5.1).
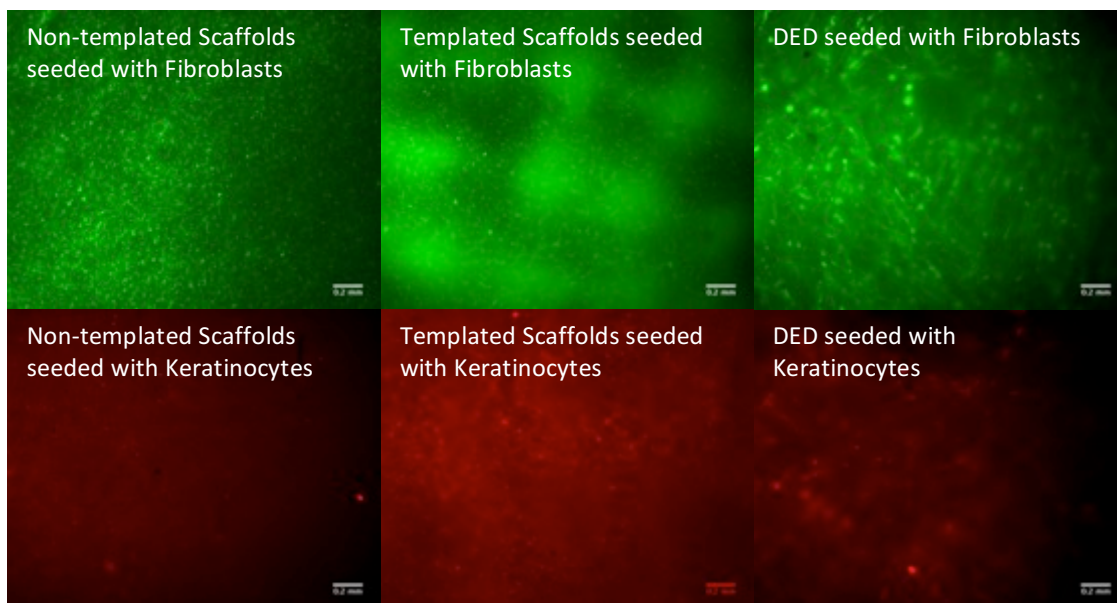
Figure 5.1: *Representative images of cell tracker fluorescence for isolated human fibroblasts (top 3 rows) between passage 4-9 and isolated human keratinocytes (Bottom 3 rows) between passage 1-4 seeded on non-templated and templated electrospun scaffolds and DED. Scale bar = 0.2 mm.*

AlamarBlue assay

Absorbances for AlamarBlue treated cells seeded on templated and non-templated scaffolds were read and the absorbance for scaffolds of each type seeded without cells were subtracted from these values for each time checkpoint of day 0, 7, 14, and 21 (Figure 5.2). Furthermore, the change in absolute values is represented as a percentage change in metabolic activity relative to day 0 from day 7, 14, and 21 (figure 5.2). This was done by subtracting the absorbance for cell seeded scaffolds after accounting for absorbance from plain scaffolds, from values obtained at day 0, which was performed after two hours following seeding of cells onto scaffolds. Additionally, we compared the

metabolic activity of the cells seeded on the electrospun scaffolds to those seeded on tissue engineered skin composites using DED as a standard. It was demonstrated that cellular constructs were viable throughout the 21-day testing period both on the scaffolds and the DED. Cellular metabolic activity in constructs containing electrospun scaffolds initially decreased from day 0 but then steadily increased from day 7 through to day 21. There were no significant differences in cellular metabolic activity when isolated cells were seeded on templated versus non-templated electrospun scaffolds. There was however a significant difference in metabolic activity seen in cells seeded on DED compared to those seeded on templated and non-templated scaffolds from day 7 to day 21.
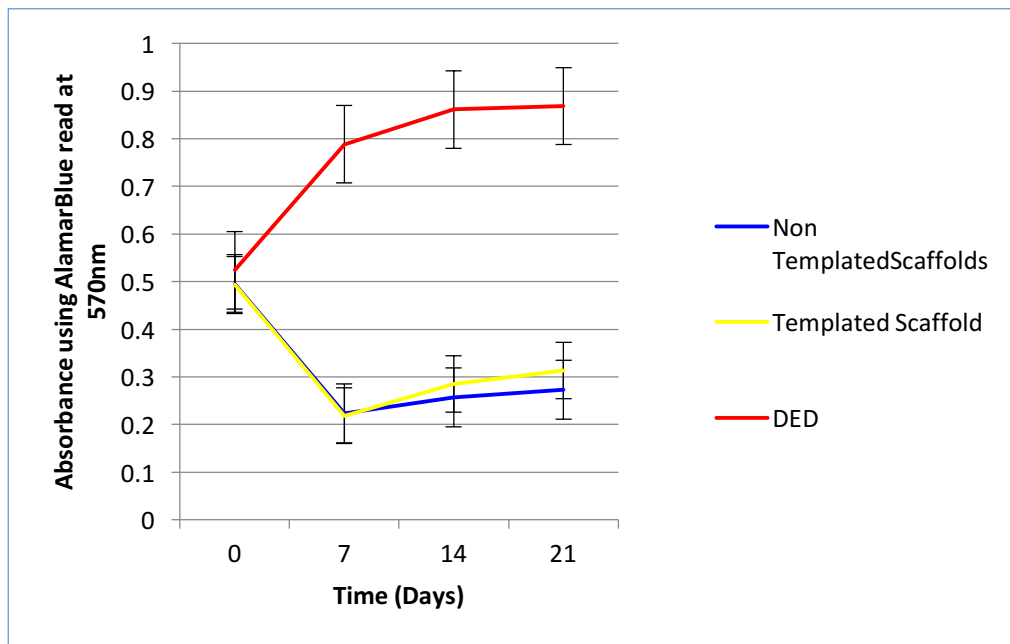
Figure5.2 *Metabolic activity of isolated keratinocytes and fibroblasts on templated and non-templated electrospun scaffolds and DED over a 21-day experimental period. Absolute change in metabolic activity over 21 days of culture, as determined by absorbance of AlamarBlue stain (Mean±SEM) (N=3)*

DAPI Staining

This was undertaken for constructs at day 7, 14, and day 21 to demonstrate the cellular attachment, proliferation and migrate along the fibres of templated trilayer electrospun scaffolds and whether there were any differences observed, with the non-templated scaffolds. DAPI imaging was also undertaken for seeded scaffolds as the negative control and tissue engineered skin composites using DED and isolated cells as the positive control at the above time check points. Good cell attachment was demonstrated on both templated and non-templated

electrospun trilayer scaffolds, which were comparable in pattern and

distribution to DED constructs (Figure 5.3).
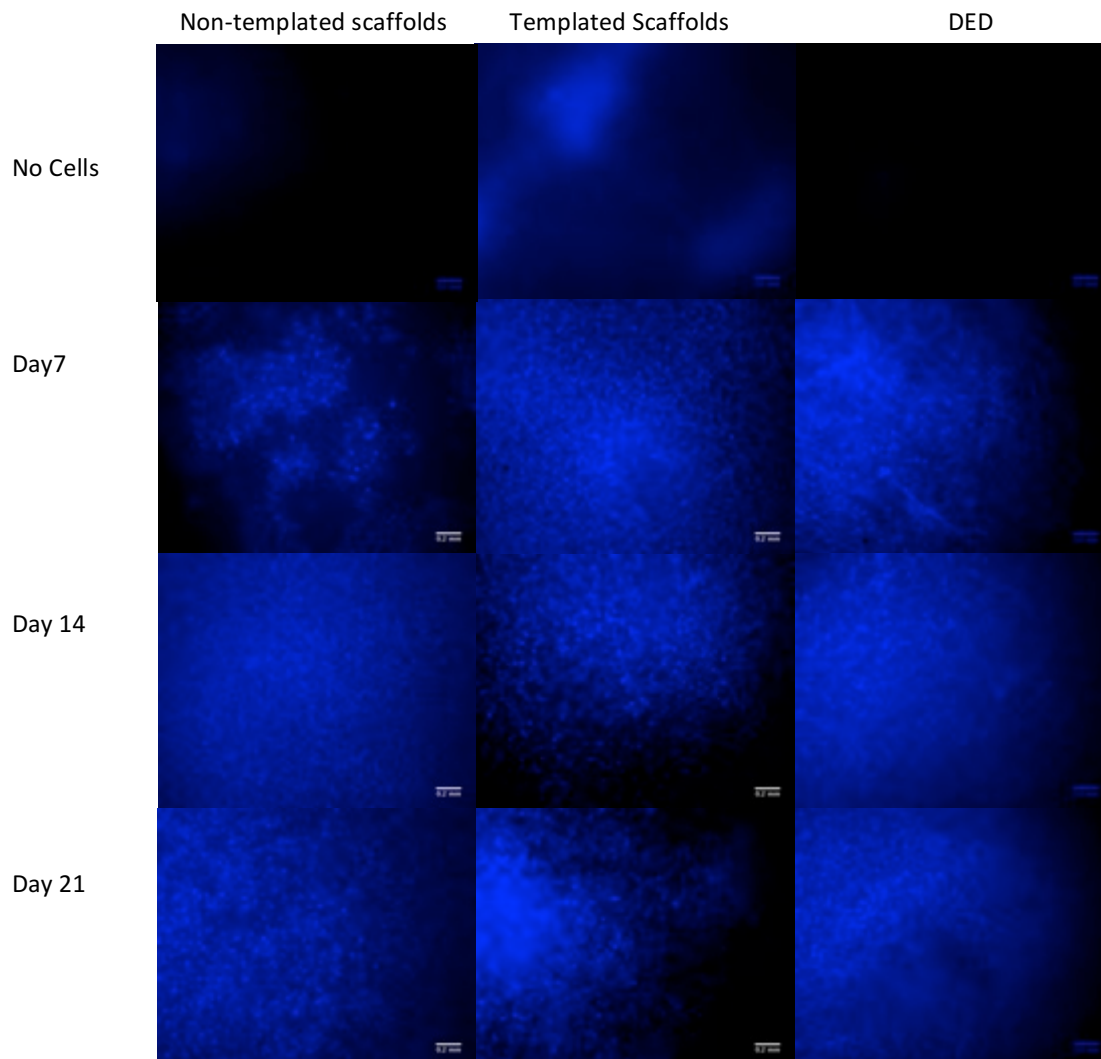
Figure 5.3 *DAPI staining of constructs (non-templated scaffolds, templated scaffolds and DED) with isolated human fibroblasts and keratinocytes. Top row also demonstrates scaffolds without cells as a negative control. Scale bar=0.2mm*

Haematoxylin and Eosin Staining.

This was undertaken on formaldehyde fixed constructs at day 7, 14, and day 21 to demonstrate consecutive cellular/scaffold architectures and any evidence of integration between the two. The cellular nuclei appear as blue black and the cytoplasm and surrounding connective tissue appear pink. Frozen sections of constructs containing cell seeded templated and non-templated scaffolds demonstrated with H&E staining, confluent cell populations on the scaffold, most predominantly seen on day 14. Furthermore, there was a distinct middle layer of PHBV that was seen as an interface between the neo epidermis the area seeded with keratinocytes and the fibroblast populated dermis (Figure 5.4 and 5.4.1).

A

Neo-epidermis with strands of keratin made from seeding isolated keratinocytes on DED

De-epitheliased dermal scaffold populated with isolated fibroblasts
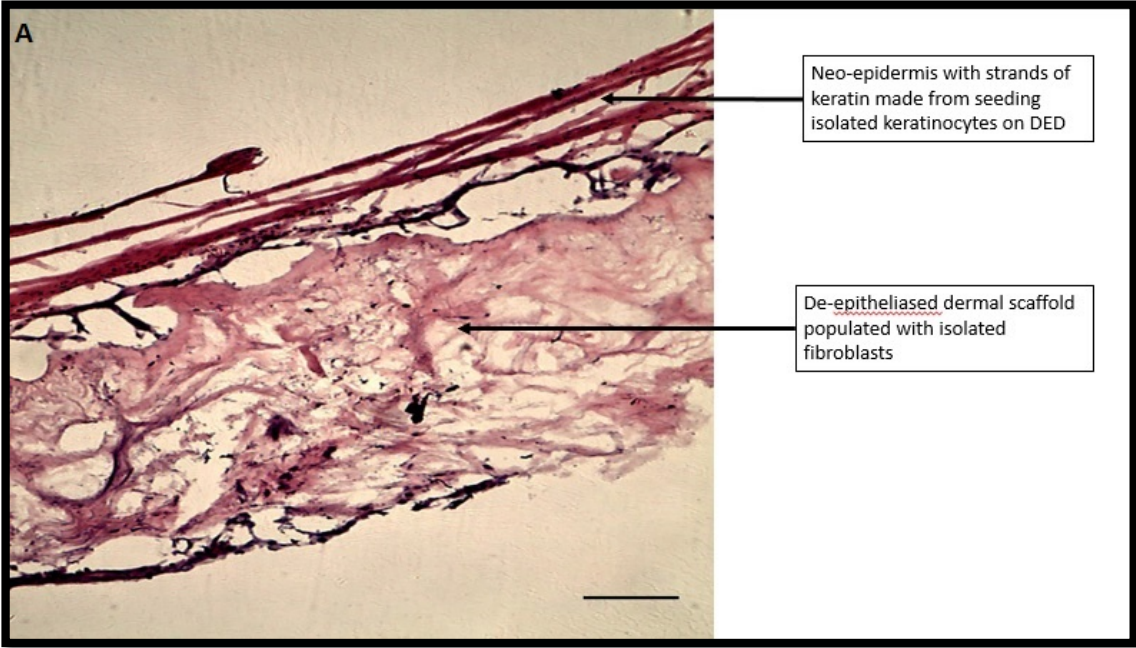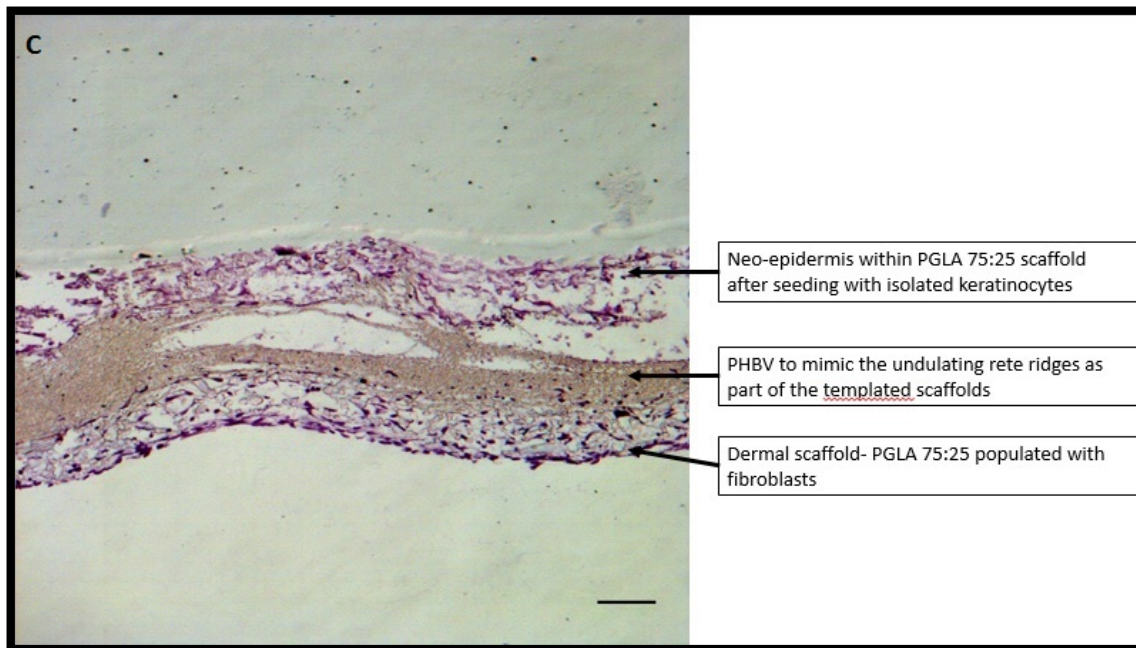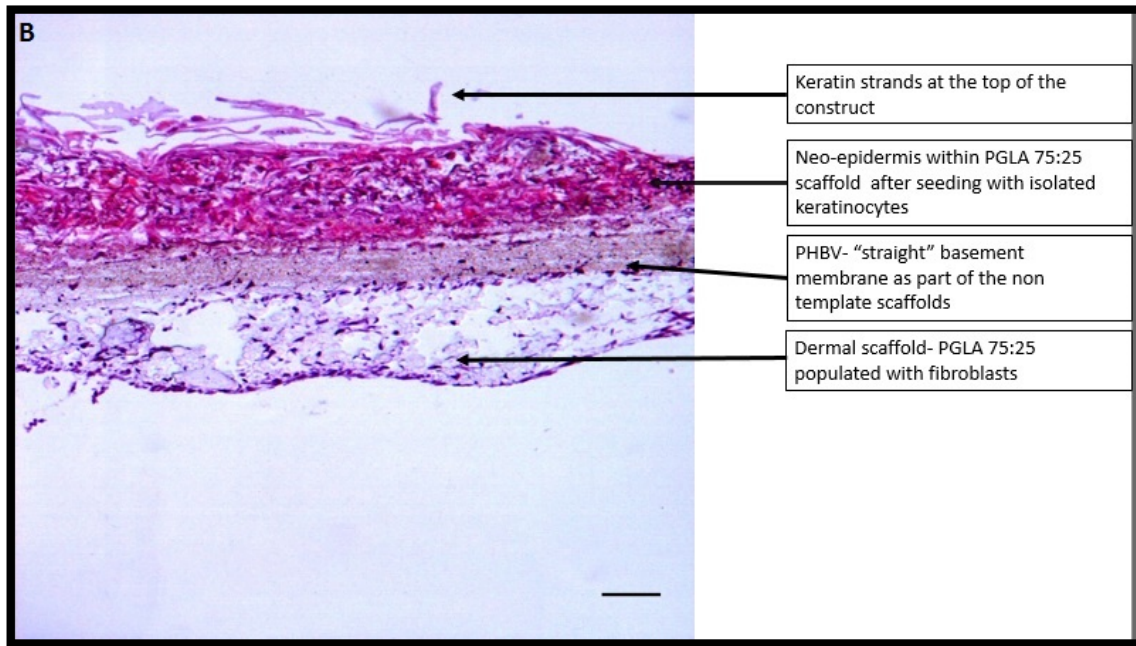
Figure 5.4 *Annotated light microscopy H&E stained sections of isolated keratinocytes and fibroblasts seeded on: A: DEDs, B: non-templated trilayer scaffolds and C: templated trilayer scaffolds over 14 days in culture. Scale bars of 0.2 mm (100x magnification).*
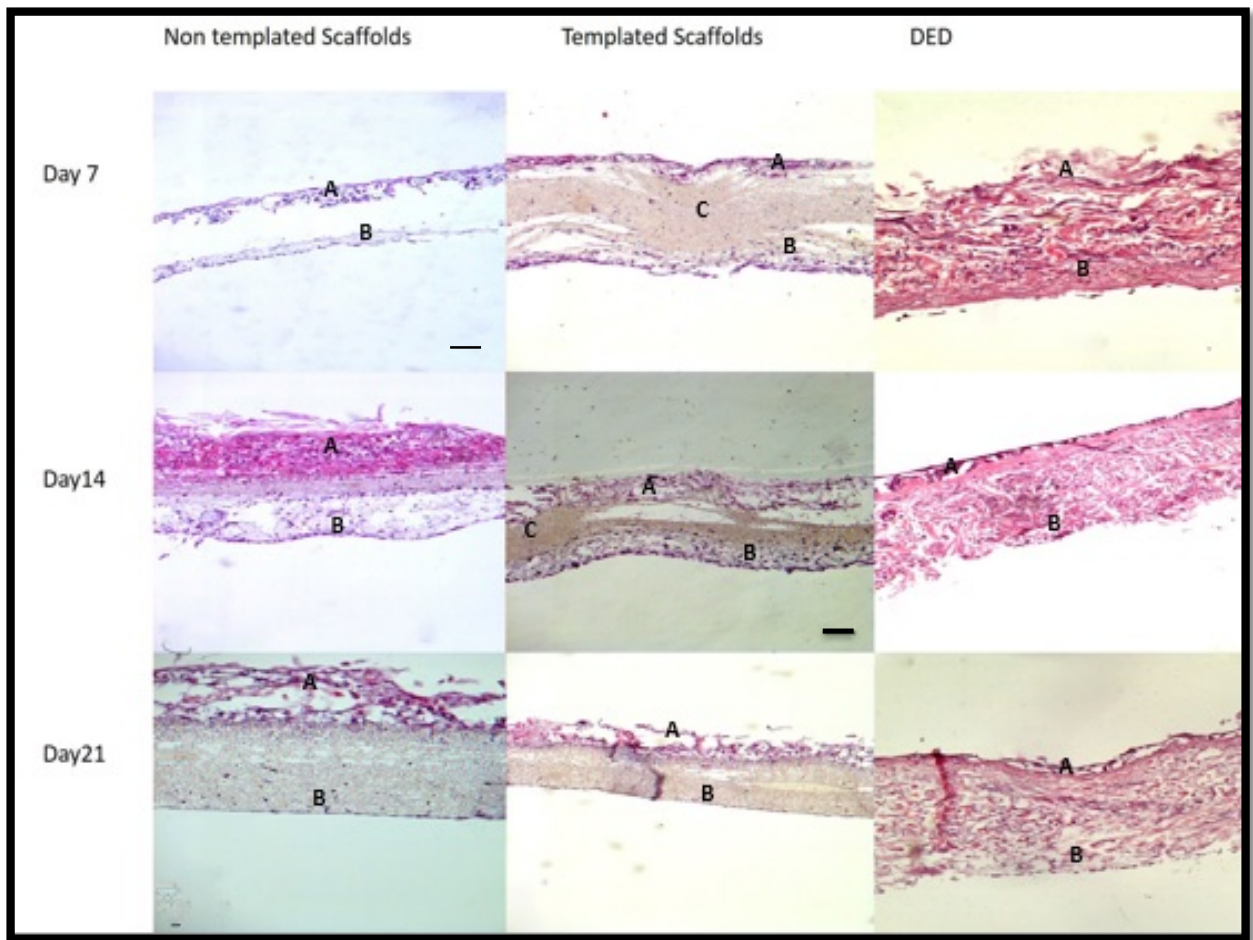
Figure 5.4.1 Representative light microscopy H&E stained sections of cells seeded on non-templated, templated and DED scaffolds. Scale bars of 0.2 mm (100x magnification) applies to all images. A=neoepidermis; B=dermal layer made of electrospun scaffold or DED, C=template on electrospun scaffold.

Sirius Red Assay

At day 7, 14, and 21 Sirius red assays were undertaken to estimate the total collagen produced overtime by the human dermal fibroblasts seeded on the templated and non-templated trilayer electrospun scaffolds (Figure 5.5). The aim was to quantify the ability of the electrospun scaffolds to support cellular function using collagen production by fibroblasts as a marker and to compare this to tissue engineered skin constructs containing DED as a standard. The absorbance of Sirus red is plotted in Figure 5.5.1 minus the absorbance of constructs containing scaffolds without cells. There was no significant difference in total collagen produced per gram of scaffold between templated and non-templated electrospun scaffolds. There were however significant differences between the collagen produced in DED constructs versus templated and non-templated scaffolds respectively, with the former producing the highest amount of collagen per gram of dry scaffold (table 8).

Electrospun scaffolds   DED

Day 7

Day 14

Day 21
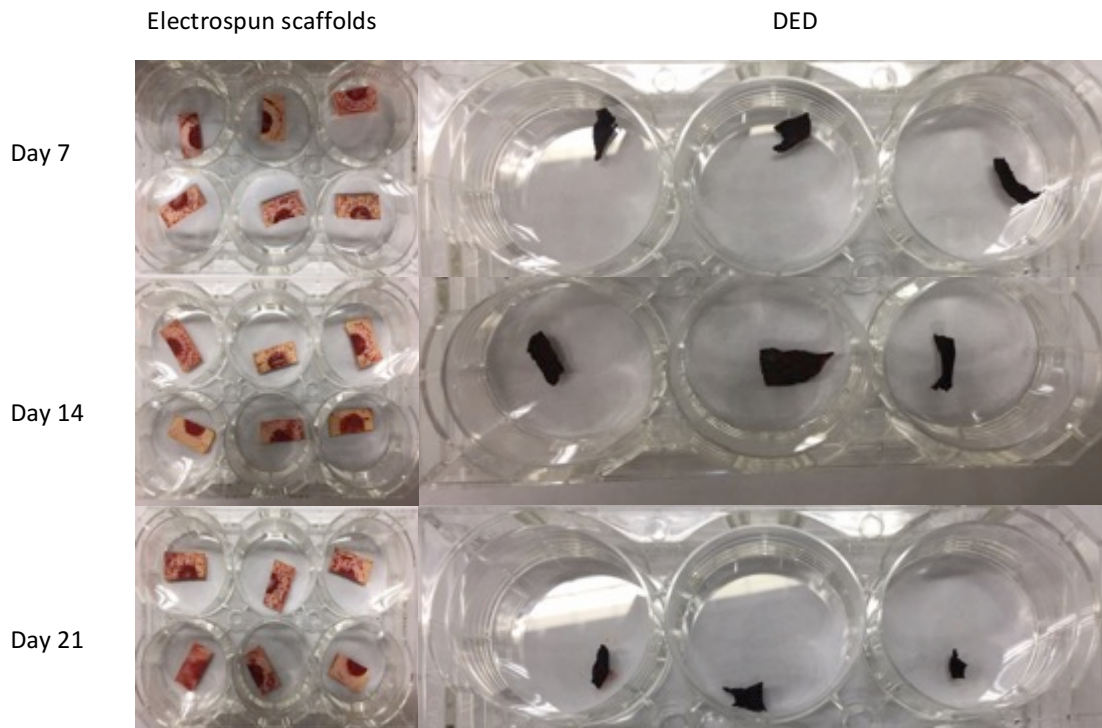
Figure 5.5 *Examples of Sirius red staining after 7, 14, and 21 days culture of isolated human fibroblasts and keratinocytes on templated (top row of 6 well plates), non-templated (bottom row of 6 well plates) electrospun scaffolds and DED.*
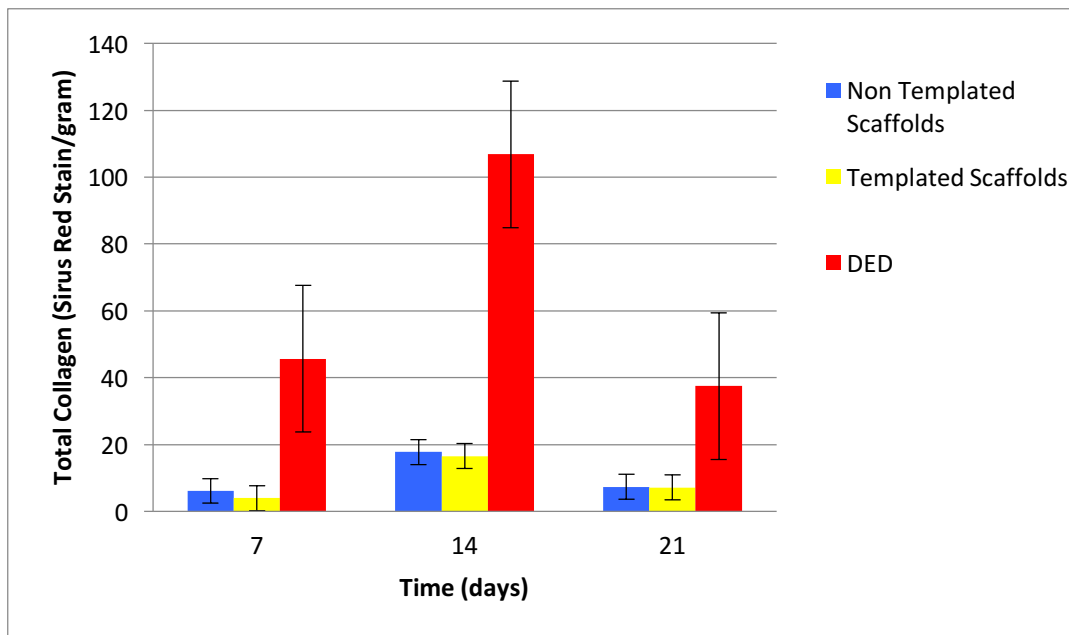
Figure 5.5.1 *Sirius red staining after 7,14, and 21 days of isolated human fibroblasts and keratinocytes cultured on templated, non-templated electrospun scaffolds and DED. (N=3±SEM).*

Day	Comparison	N	2 tailed P value
7	DED vs. Templated Scaffolds	3	0.0033
	DED vs. Non-templated Scaffolds	3	0.0040
14	DED vs. Templated Scaffolds	3	0.0020
	DED vs. Non-templated Scaffolds	3	0.0020
21	DED vs. Templated Scaffolds	3	0.0178
	DED vs. Non-templated Scaffolds	3	0.0190

Table 4: *Results of unpaired T test for Sirus Red absorbance values comparing cell seeded constructs on DED and electrospun scaffolds at day 7, 14, and 21. Significance was taken as p<0.05.*

5.3 Culture of Skin Explants on Templated versus Non-templated Trilayer Electrospun Scaffolds

This experiment involved culturing skin explants onto templated and non-templated trilayer electrospun scaffolds to determine if cells would migrate from the cut edges of the explants onto the fibres of the scaffolds. A fixed area (5x5 mm^2) piece of split thickness skin was minced very finely to create these skin explants. The idea here was to increase the surface area of the cut edges giving the epithelial cells more opportunity to migrate from the skin pieces. Furthermore, we also aimed to compare if there was any difference between cellular migration and proliferation on templated versus non-templated scaffolds. After 48 hours, the skin explant-scaffold constructs were raised to an air liquid interface and cultured for 7, 14, and 21 days respectively.

AlamarBlue^{TM} assay

Absorbance for skin explants cultured on templated scaffolds and non-templated scaffolds were read and the absorbance for scaffolds of each type with no skin explants were subtracted from these values. The change in absolute values is represented as a percentage change in metabolic activity relative to the control at every time check point namely day 7, 14, and 21. Furthermore the metabolic activity of the skin explants seeded on the electrospun scaffolds were

compared to tissue engineered skin composites using DED as the standard. It was demonstrated that the skin explants were viable throughout the 21-day testing period both templated and non-templated scaffolds.

Cellular metabolic activity in constructs containing both templated and non-templated electrospun scaffolds like those of the cell-seeded constructs decreased from day 0 to day 7 but then steadily increased through to day 21 (Figure 5.6). There was no significant difference metabolic activity when skin explants were seeded on templated versus non-templated electrospun scaffolds. There was however a significant difference in metabolic activity seen in cells seeded on DED compared to those seeded on templated and non-templated scaffolds respectively (table 9). Furthermore, there were no significant differences in % metabolic change from day 0 to day 21 between skin explants cultured on templated and non-templated electrospun scaffolds.
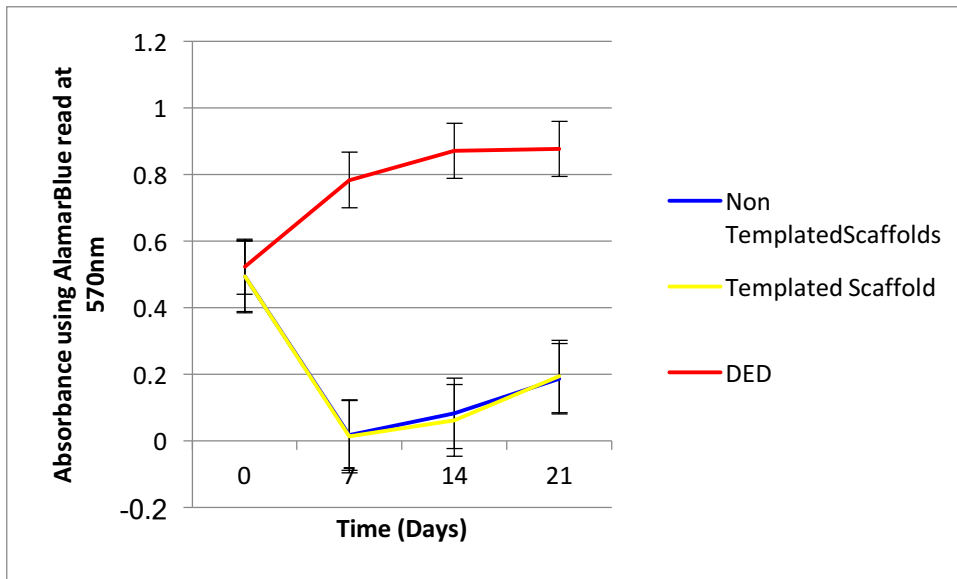
Figure 5.6 *Metabolic activity of skin explants cultured on templated and non-templated electrospun scaffolds and DED over a 21-day experimental period. Absolute change in metabolic activity over 21 days of culture, as determined by absorbance of AlamarBlue stain (mean±SEM) (n=3)*

Day	Comparison	N	2 tailed P value
7	DED vs. Templated Scaffolds	12	<0.0001
	DED vs. Non-templated Scaffolds	12	<0.0001
14	DED vs. Templated Scaffolds	9	<0.0001
	DED vs. Non-templated Scaffolds	9	<0.0001
21	DED vs. Templated Scaffolds	3	<0.0001
	DED vs. Non-templated Scaffolds	3	<0.0001

Table 5: *Results of unpaired T test for AlamarBlue absorbance values comparing skin explant cultured constructs on DED and electrospun scaffolds at day 7, 14, and 21. Significance was taken as p<0.05.*

DAPI Staining.

This was undertaken for constructs fixed at day 7, 14, and day 21 to determine firstly if cells were able to leave the cut edge of the skin explants and migrate along fibres of the electrospun scaffolds and whether there were any differences observed, if any with the non-templated scaffolds. DAPI imaging was also undertaken for tissue engineered skin composites using DED the above time check points as a comparison. Cells were seen to be leaving the surrounding area around the skin explant pieces, however compared to tissue engineered skin constructs using DED there was not as much cellular migration and proliferation observed. It is appreciated that the cells would take time to leave the explants and migrate along fibres; therefore, a comparison at day 21 was undertaken. These images still do not demonstrate a significant amount of cellular migration compared to day 21 DED samples (Figure 5.7).
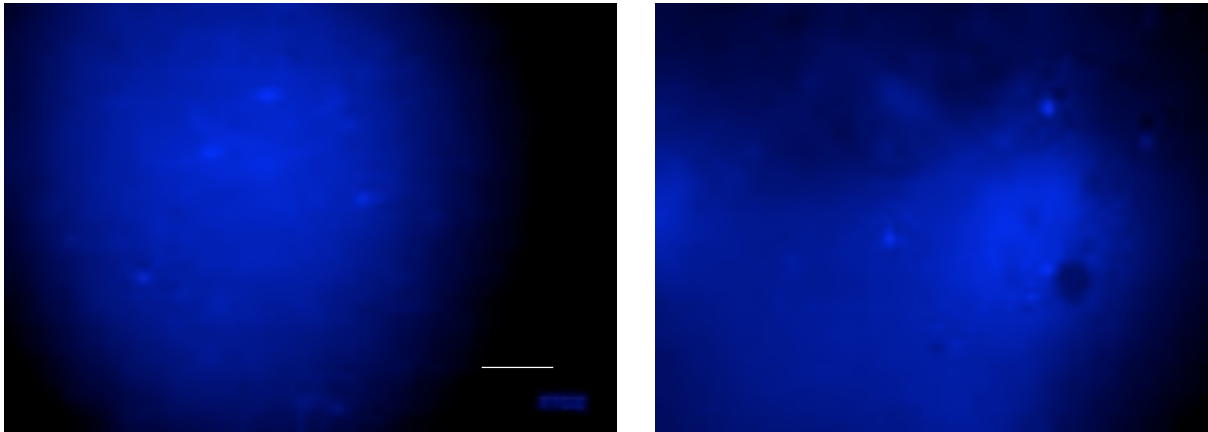
Figure 5.7 DAPI staining of constructs (non-templated scaffolds(left), templated scaffolds (right) cultured with skin explants at day 14 illustrating little to no cellular nuclei of cells migrating from the skin explant onto the scaffold. Scale bar =0.2mm.

Haematoxylin and Eosin Staining.

These were undertaken on fixed constructs at day 7, 14, and day 21 to demonstrate consecutive cellular/scaffold architectures. The cellular nuclei appear as blue black and the cytoplasm and surrounding connective tissue appear pink. Frozen sections of constructs containing skin explant cultured on non-templated (Figure 5.8.) and templated (5.8.1) scaffolds demonstrated with H&E staining, very little cellular outgrowth from the cut edges of the skin explant. Due to the lack of cellular migration and integration within fibres of the scaffold it can be observed that the scaffolds are visibly devoid of these and in most cases have lost their distinctive trilayer architecture during the process of H&E staining. There is also a very obvious difference in cellular integration and

ECM production between constructs seeded with isolated cells (both

electrospun scaffolds and DED) and those cultured with skin explants with the

former having definitive evidence of an epidermal and dermal layer (figure

5.4.1).
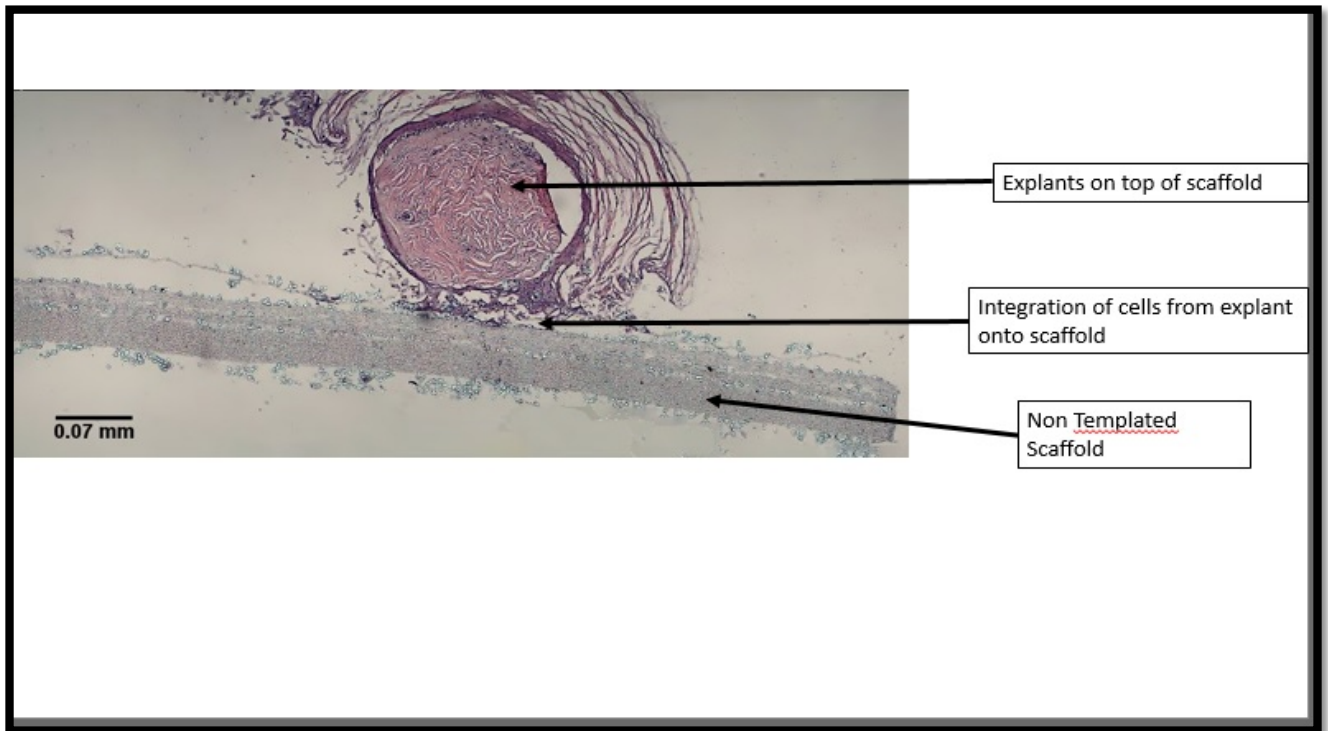
Figure 5.8 Annotated panoramic light microscopy H&E stained sections of skin explants seeded on non-templated trilayer scaffolds over 21 days in culture. Scale bars of 0.07 mm (100X magnification).
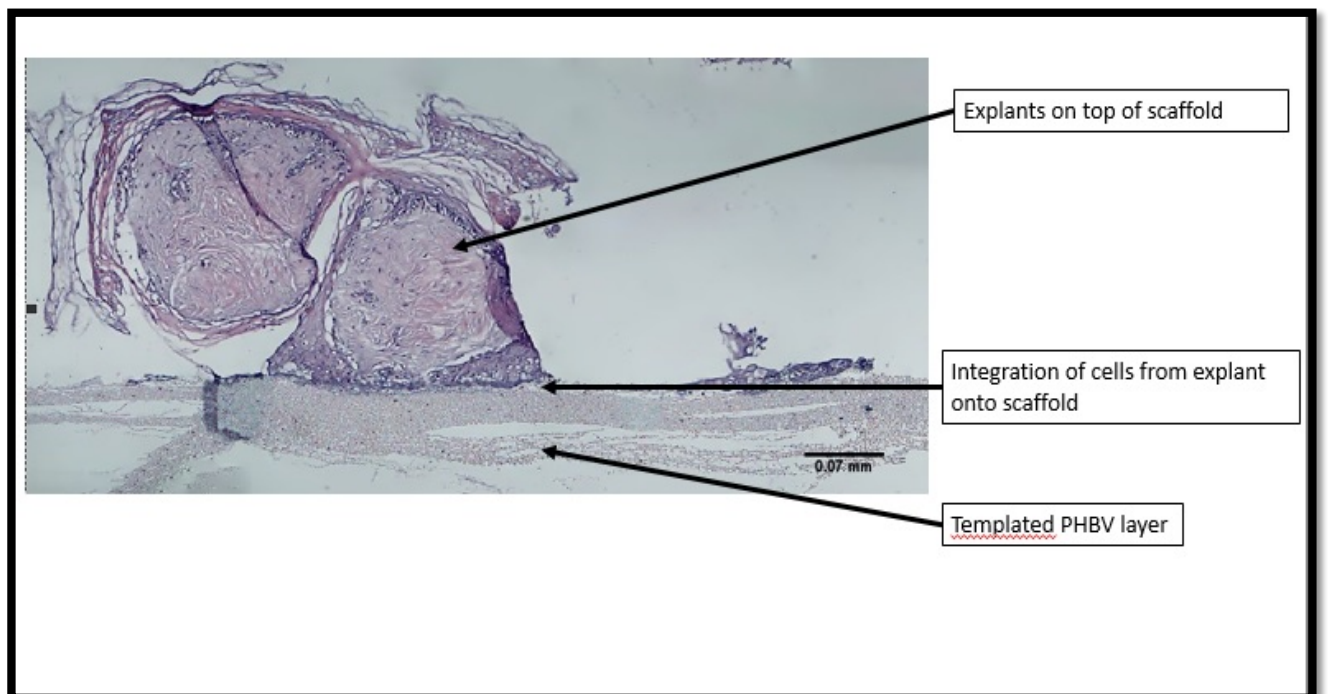
Figure 5.8.1 Annotated panoramic light microscopy H&E stained sections of skin explants seeded on templated trilayer scaffolds over 21 days in culture. Scale bars of 0.07 mm (100X magnification).

Sirius Red Assay

At day 7, 14, and 21 Sirius red assays were undertaken to estimate the total collagen produced overtime by the skin explants seeded on trilayer electrospun scaffolds (Figure 5.9). The aim was to quantify the amount of collagen deposited by fibroblasts that have migrated and proliferated along the fibres of the electrospun scaffolds from the skin explants. This was compared to constructs containing DED and isolated cells. There was no significant difference in total collagen produced per gram of scaffold between templated and non-templated electrospun scaffolds (Figure 5.9.1). However, comparing skin explant constructs to isolated cell seeded with DED tissue engineered skin) there were significant differences in collagen produced with the latter being higher at all time checkpoints (table 10).

Figure 5.9 *Examples of Sirius red staining after 7, 14, and 21 days culture of skin explants on templated (top row of 6 well- plates) and non-templated (bottom row of 6 well plates) electrospun scaffolds.*
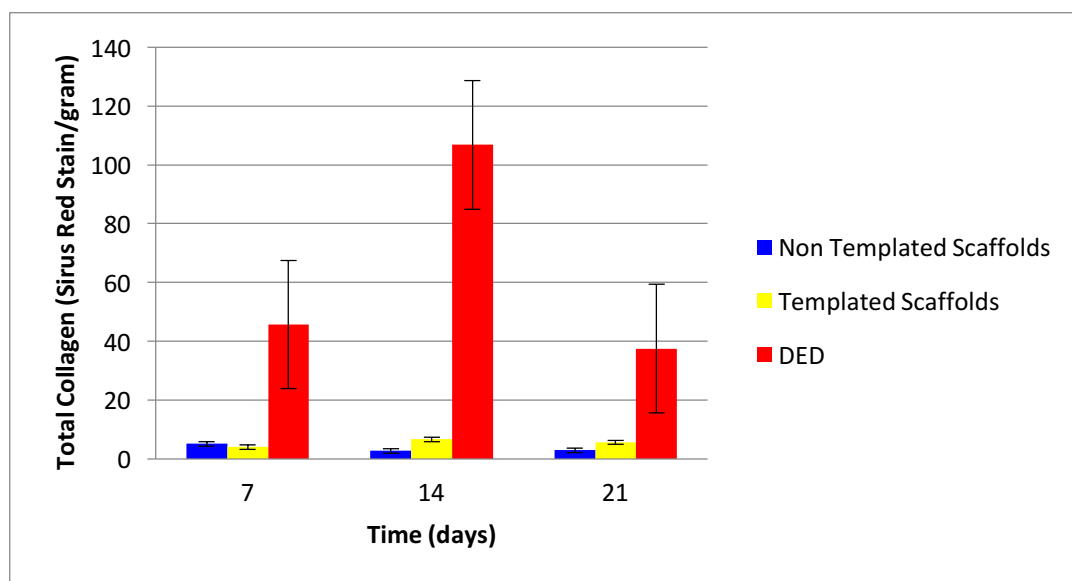
Figure 5.9.1 *Sirius red staining after 7, 14, and 21 days of skin explants cultured on templated, non-templated electrospun scaffolds and tissue engineered skin constructs containing DED. (N=9±SEM).*

Day	Comparison	N	2 tailed P value
7	DED vs. Templated Scaffolds	3	0.0033
	DED vs. Non-templated Scaffolds	3	0.0037
14	DED vs. Templated Scaffolds	3	0.0012
	DED vs. Non-templated Scaffolds	3	0.0010
21	DED vs. Templated Scaffolds	3	0.0149
	DED vs. Non-templated Scaffolds	3	0.0113

Table 6: *Results of unpaired T test for Sirus Red absorbance values comparing skin explant cultured constructs containing DED and electrospun scaffolds at day 7, 14, and 21. Significance was taken as p<0.05.*

5.4 Summary

The aims of the first set of the experiments in this chapter were to firstly determine if cells would be able to proliferate along the fibres of the templated scaffolds and function so as to start formation of an extracellular matrix. It is known that electrospun scaffolds are cell conductive, however the idea of investigating the effect of templating scaffolds so as to provide a stem cell niche is novel. Furthermore, we wanted to extend this idea to investigate if these modified electrospun scaffolds had any effect on the migration of cells from the cut edges of skin explants. Fluorescence staining using cell tracker green and red confirmed that isolated human keratinocytes and fibroblasts cells were present on each scaffold prior to raising them to an air liquid interface. In general, no significant differences were noted in the ability for templated electrospun scaffolds to support cellular migration and proliferation as compared to non-templated trilayer scaffolds. For both templated and non-templated electrospun scaffolds it was noted that there was a significant drop in cellular viability as demonstrated by AlamarBlue absorbance readings from day 0 to day 7. This could be explained by the fact that on Day 0, cells are most populous on the scaffolds whereas on day 7, some would have died and not been supported on the fibres. This contrasts the native human dermal scaffold provided for by the DED, which supports the cells from the start of seeding. Furthermore, some

cells would have fallen between the micropores of the PGLA fibres onto the bottom of the 6 well plate between day 0 and day 7 hence account for the reduction in numbers of cells and overall metabolic activity of the constructs containing electrospun scaffolds. Following day 7 it is observed that metabolic activity increases until day 21 in the constructs containing both templated and non-templated scaffolds. There were no significant differences between these two scaffolds during this time however constructs containing DED showed as expected significantly higher amounts of metabolic activity. Interestingly when % metabolic activity of scaffolds is compared relative to day 0, there seemed to be a significant difference in templated versus non-templated scaffolds suggesting that over time and longer culture periods, this scaffold architecture was more conductive to cellular migration and proliferation.

Furthermore, it was demonstrated that cells were confluent over -and distributed along the fibres of both the templated and non-templated electrospun scaffolds, which were comparable to those on DED constructs. This finding was also supported by H&E staining of histology sections. Additionally, this technique allowed us to better visualize the topography of the electrospun dermal analogue, which included the basement membrane and the niches. It is shown that keratinocytes populated the microporous side containing PGLA (75:25) and produced strands of keratin whereas the fibroblasts occupied the

other side of the PHBV basement membrane layer. Additionally, there were no notable differences in the ability of the electrospun scaffold be it templated or non-templated to support the production of an extracellular matrix containing collagen. This ability remained significantly higher in constructs containing DED however there was evidence of some fibroblast migration and proliferation to produce collagen in constructs containing electrospun scaffolds. The data presented thus far highlights the fact that the templated scaffolds supports the proliferation and migration of isolated human fibroblasts and keratinocytes. Another aim of the latter experiments in this chapter was to determine if cells from the cut edge of skin explants would migrate and proliferate along the fibres of the trilayer templated electrospun scaffolds. This is in keeping with trying to develop a sustainable off the shelf technique for dermal substitution that does not rely on expanding isolated patient's cells in the lab. The use of isolated cells as part of a skin substitute is a tried and tested technique in skin reconstruction, which has never made it into the routine of reconstructive reach for the plastic surgeon. The results demonstrate that there were no significant differences in the metabolic activity of constructs containing templated and non-templated trilayer scaffolds when cultured with skin explants. There was a decrease in metabolic activity from day 0 to day 7, which slowly increased until day 21. This was a similar picture for the cell-seeded constructs however the decreases in

cellular proliferation in the skin explant samples were more pronounced. This finding was supported by the fluorescent images using DAPI staining that illustrated very little outward migration of cells from the cut edges of the explants in comparison to cell seeded constructs and the standard tissue engineered skin constructs containing DED. Additionally, the skin explants did not show convincing evidence that cells from their cut edges integrated with the electrospun scaffolds as demonstrated by H&E staining. Furthermore because of the lack of cellular proliferation and ECM deposition on these skin explant constructs, as expected the histology process resulted in destruction of electrospun scaffold architecture and topography. This finding was also reflected in the assessment of collagen deposition quantified by Sirius Red Assays. Although there was some collagen production, we postulate that this was mainly due to the natural collagen contained in the skin explants themselves as opposed to fibroblast integration and function on the trilayer scaffolds, one limitation of our study was that it was very difficult to determine the amount of collagen that were present in the skin explants as a control. This would involve undertaking Sirus red on a fixed sample and assume that this sample was representative of all the repeats used, however there would be significant variation in the various batches of skin with regards to the amount of collagen in the dermis and in between patients to use this technique as a

standard. . This is evidenced in Figure 5.9 which highlights a concentration of the Sirius red stain on the actual skin explants as opposed confluent staining on the dermal scaffold which was observed in the cell seeded electrospun constructs and tissue engineered skin models containing DED (Figure 5.5). The data presented here also suggests that the use of isolated cells on trilayer electrospun scaffolds resulted in constructs with significantly better overall cellular proliferation, integration and function as opposed to using skin explants as a cell source. The cells contained in the skin explants are viable throughout the experimental time period and in theory have the ability to migrate from the cut edge of the skin explants onto the fires of the electrospun scaffold. This technique as mentioned in the introduction is taken from the principles of Meek Grafting, where small squares are cut from split thickness skin and placed onto a wound bed. This results in cellular migration from the edge of the grafted squares of split thickness skin and re-epithelisation of the wound bed. However, using that same principle, replacing the wound bed with the electrospun scaffold reveals that there is not enough migration of cells from the cut edges of the skin explants along the fibres of the electrospun scaffolds which would ultimately serve to augment wound healing. During the experimental process, from a practical viewpoint, it was very challenging to keep the finely dissected skin explants in contact with the scaffold at all times, which is needed for cellular

migration. Firstly, there was a tendency for some of the pieces to float in the media. Furthermore, media changes and assessment of cellular viability using AlamarBlue assays would often disturb the explants. Despite these disturbances being only 3 times per week and performed in a slow and careful manner so as to not disturb the constructs, there were many instances where the quantity of skin explants was noted to be less compared to the start of the experiment. As this technique is modelled on the successful clinical application of meek skin grafting, this led us to believe that our skin explant model needed to be refined. There should be a reasonable explanation for this lack out outward cellular growth. Therefore, we postulated that there were two main factors that could contribute to this lack of outward cellular migration, firstly the properties of the skin explant themselves and the interaction between the skin explants and scaffolds.

With regards to the skin explants themselves, it is known that the keratinocytes and fibroblasts without the appropriate chemical signalling will not be stimulated to leave the skin explant and migrate onto the fibres of the electrospun scaffold. To migrate over a wound site, the keratinocytes must disengage their desmosome/hemidesmosomes junctions and re-organize their cytoskeleton. As re-epithelization continues keratinocytes once again by receiving the correct signalling result in a cessation of this migration and

reversion to their normal phenotype, using hemidesmosomes to attach to the basement membrane and the underlying dermis through collagen fibrils(210).

Foundation work in cell biology revealed the necessity to co-culture fibroblasts with keratinocytes in order to stimulate growth and proliferation(211). Keratinocytes whilst in culture instruct fibroblasts to synthesize and secrete growth factors and cytokines like keratinocyte growth factor (KGF)/fibroblast growth factor-7 (FGF7), IL-6, and GM-CSF(212). This in turn stimulates the migration and proliferation of keratinocytes and ultimately wound epithelization resulting in this double paracrine loop. In the absence of these signalling proteins migration of these cells does not occur. This could be a possible explanation of the lack of migration of keratinocytes and fibroblasts in our novel tissue engineered skin model containing electrospun scaffolds and skin explants. Additionally, cells will only migrate out of the explants where it is in direct contact with the fibres of the electrospun scaffolds. This will be increased in constructs in which isolated cells are used as compared to skin explants as there will be greater number of contact points between the scaffold fibres and isolated cells versus skin explant and fibres as the cells are still retained in the block of tissue. Additionally, the interaction between the skin explants and electrospun scaffolds in vivo is very different to that which occurs with the model of meek grafting in the clinical environment, which this concept

is based on. Meek grafts when placed on wound beds are often secured to it using tissue glue and layers of initially non-adherent, absorbent and elastic dressings to ensure that the grafts are held in place and in the right orientation. These wounds are not disturbed for five days initially to allow good contact to occur between the meek graft squares and the wound bed and thereby cellular migration and proliferation. In our in vitro constructs the skin explants are simply placed on top of the electrospun scaffolds without any method to secure their contact. Furthermore, media changes which are required result in physical disturbances to this contact. Therefore, we need to refine this in vitro skin explant model to allow for a more secure contact between the skin explants and the electrospun scaffolds and investigate potential strategies to increase the cellular migration and proliferation from the cut edges of the skin explants.

Chapter 6

Investigation of approaches to achieve adhesion of skin pieces to scaffolds to increase the outgrowth of cells into the scaffolds.

6.1 Introduction

Experiments from Chapters 3 and 4 illustrate that simply placing skin explants directly onto an electrospun scaffold *in vitr*o does result in some skin cell migration from the cut edges of skin explants however not at a rate that would be appropriate for use of this product in the clinical environment. The ultimate goal is for the electrospun scaffold is to form part of a new dermis, which is to be populated by cellular outgrowth from the skin explants. This chapter aims to assess potential strategies to aid retention of the skin explants onto the electrospun scaffolds and to improve the migration of skin cells along the scaffold fibres.

The strategies tested were:

1. The use of fibrin, a clinically used adhesive to aid retention of the skin explants onto the electrospun scaffolds.

2. The use of explants with less dermis to determine is this has an effect on cellular migration onto the scaffolds

3. The use of an additional scaffold overlying the skin explants to aid retention of the pieces on the scaffold.

The aim is to achieve a level of cellular migration similar, or close to that of seeding isolated cells on electrospun scaffolds. In terms of wound healing, fibroblasts begin proliferating to form type III collagen from the fourth day following injury for up to 2-4 weeks thereafter to replace dermal breaches(213). At the same time, keratinocytes also begin to migrate. Therefore, these constructs should have some evidence of migrating and proliferating cells from at least the 7 to 14-day time checkpoints.

As outlined in the introduction, the use of skin explants as a source of skin cells to aid wound healing is a successful technique employed in Meek grafting. This is due to the technique itself and the presence of a suitable wound bed containing the appropriate architecture and signalling molecules that are essential for fibroblast and keratinocyte migration and proliferation. The surgical technique of Meek grafting has been refined over time, but its principle remains the same to ensure clinical success and reproducibility i.e. re-epithelization of the wound bed. In this method, the skin explants are placed onto the wound bed and secured with either glue or sutures followed by layers of dressings. This is needed to prevent shear forces that can disrupt the fibrin that is formed between the grafts and the wound bed as part of the initial phase of graft take. This adhesion to the wound bed is a vital part of skin graft take, as only when this occurs, the keratinocytes in the basal layer of the epidermis show

high proliferation rates which serves to stimulate growth factor excretion and conversely further proliferation of cells(213). Furthermore, fibrin acts as a scaffold for skin cells to migrate along and thereby re-epithelize. As outlined previously, it was found that due to the *in vitro* conditions of the construct, skin explants were lost during media changes and AlamarBlue measurements during the experimental time period. We postulated therefore that one of the reasons why there was lack of migration of skin cells onto the fibres of the electrospun scaffold was because the explants were not in contact with the scaffolds at all times. Furthermore, fibrin was not present between the skin explant and the electrospun scaffold, which is what would normally occur when explants are placed onto a wound bed. In order to address these, this chapter aims to assess various strategies to aid retention of the skin explants onto the electrospun scaffolds thereby optimizing the migration of skin cells along the scaffold fibres. The first strategy assessed the use of fibrin to fix the skin explants onto the electrospun scaffold. We investigated this using both laboratory made fibrin gels of varying fibrin concentrations, and a clinically available product TISSEEL™: (Baxter Healthcare Corp, Deerfield, IL, USA). Furthermore, we aimed to determine whether or not the dermal thickness of the skin explants had any influence, if any, in determining how quickly skin cells migrate from the cut edges of the skin explants. Finally, as our last optimization strategy we placed

the skin explants between two layers of electrospun scaffold. The second layer acted somewhat like a dressing that would normally be put onto a skin graft once it has been applied to the wound bed to keep the skin explants in place during the 21-day experimental time period.

6.2 Fibrin

Fibrin is a meshwork of insoluble protein fibres that is formed as a result of the activation of the coagulation cascade. The end point of the coagulation cascade is the production of a serine protease called thrombin from its precursor form prothrombin. Thrombin converts fibrinogen to fibrin. Fibrinogen is a soluble glycoprotein, which when exposed to thrombin results in cleavage of two small peptides (fibrinopeptides A and B). This results in the formation of protofibrils that aggregate to form insoluble fibrin fibres. The fibrin network is further stabilized by activated factor XIII, as the network can undergo early degradation by a serine protease called plasmin(214)(148). This fibrin network has multiple functions. Firstly, it reinforces the initial platelet plug by trapping platelets, erythrocytes, and other proteins to stop bleeding. It also serves as an initial template for promoting cellular migration and proliferation as it is filled with cytokines and growth factors which promote re-epithelization, angiogenesis, and collagen deposition(215). Furthermore, fibroblasts from surrounding

uninjured tissue are stimulated both by growth factors from within the fibrin network and the network itself to migrate towards the matrix. Fibroblasts express integrin receptors, proliferate, and produce extracellular matrix components to replace the temporary fibrin network. Keratinocytes are also stimulated by the fibrin network to detach themselves from the surrounding healthy wound edges and migrate towards it- instituting the process of re-epithelization(216)(16).

The concentration of thrombin at the time of fibrin clot formation has a significant influence on fibrin clot structure(217). Extremely low concentrations of thrombin (<1nM, < 0.1 U/mL) produce turbid clots composed of thick, loosely woven fibrin strands. Higher concentrations of thrombin produce relatively thinner fibrin clots composed of tightly packed fibrin strands(218). Thrombin becomes inactivated once the fibrin clot has formed. Fibrin is attractive for use in tissue-engineered skin. Firstly, it serves as a stimulus to promote cell adhesion, migration and proliferation. Additionally, fibrin precursors thrombin and fibrinogen can be easily isolated from patient's blood thereby creating a custom autologous treatment. In this regard, the fibrin clot should have a porous structure to facilitate cellular movement and function but at the same time be mechanically strong to combat shear forces that can occur between the skin

explants and the electrospun scaffolds. Furthermore increasing the fibrinogen concentration results in a clot with an increased tensile and adhesion strength(219).

We tested three concentrations of thrombin and three concentrations of fibrinogen. The first concentrations tested were based on that used in previous studies performed in the MacNeil laboratory on corneal explants. Ortega et al used a 1:1 mixture of fibrinogen at a concentration of 18.75mg /ml and thrombin at a concentration of 2.5 U/ml, which was found to adhere the limbal explants onto the electrospun scaffold and at the same time facilitated outward cellular migration. Fibrin as mentioned in the introduction is used clinically as a haemostat, sealant, and an adhesive. The clinical applications of sealants and adhesives were chosen as models for our concentrations. Fibrin is used as a haemostat in cases of bleeding which is not controllable by suturing or cauterization of vessels e.g. solid organ lacerations. In this case the thrombin concentration used is often greater than 500 I.U/ml, this high concentration of thrombin would result in a tightly compact fibrin clot that sets instantly, which is a requirement of its function as a haemostat. However, this would not be suitable for our *in vitro* studies. Other thrombin/fibrinogen concentrations used are based on the approximate concentrations from these clinical products.

Thrombin/fibrinogen concentration of 2.5-6.5 I.U / 67-86.5mg/ml is based on the use of fibrin as an adhesive for skin graft attachment at burn wound debridement sites. This is marketed commercially as Artiss(220).

The key feature that makes it a useful adhesive is the relatively low thrombin concentration of 4.5 IU (range 2.5 to 6.5) that allows at least up to 60 seconds to position the grafts or flaps before the fibrin starts to set. The fibrinogen concentration in this product ranged from 67 to 106 mg/ml with an average of 86.5mg/ml. The fibrinogen concentrations that we chose (67mg/ml and 86.5mg/ml) reflect the lower and average concentrations of fibrinogen present in this product. These concentrations were postulated to be beneficial in our *in vitro* studies, as it would allow some time to manipulate the skin explants onto the electrospun scaffold fibres before they were adhered in place.

Tisseel is another product that we tested in its ability to fix skin explants onto the electrospun scaffold and facilitate cellular migration from the cut edge of the skin explants. This is marketed both as a sealant for vascular and cardiac procedures and as a haemostat for cases where suture or cautery of vessels are not appropriate. This product has a thrombin concentration ranging from 400 – 625 units/mL and a fibrinogen concentration ranging from 67 – 106 mg/mL(221). As this preparation is FDA approved, widely available, and used within the United Kingdom, it was felt that it would be beneficial to test its

suitability in our *in vitro* constructs as this would make clinical translation using fibrin more realistic.

Additionally, we wanted to assess the effect of dermal thickness on skin cell migration from the cut edges of skin explants. The earliest studies that examined the effect on dermal thickness on successful skin graft take were undertaken by an American surgeon called Earl Padgett (50). He divided skin grafts into four standardized thicknesses: Thiersch grafts (0.008–0.010 inches; 0.2–0.25mm), STSGs (0.012–0.016inches; 0.3–0.4mm), three-quarter skin grafts (0.018–0.022inches; 0.45–0.55mm), and full thickness skin grafts (0.030 –0.038 inches; 0.75–0.95mm)(50).

He concluded that using grafts with less dermis was suitable for granulating wound bases and grafts with more dermis for wounds that would require more substantial coverage to avoid contractures e.g. over joint surfaces. The dermal thickness of a graft is dependent on the technique used, the quality of the patient's skin (age, drugs and other co-morbidities), and the chosen donor site. Areas like the inner arm and thigh have thinner dermis and result in such grafts being thinner than grafts from donor sites like the abdomen, back and outer thigh.

Furthermore, the use of an air-powered dermatome has standardized graft thickness to some extent as the desired thickness can be set in thousandths of

an inch. However, the pressure placed on the instrument by the surgeon has an influence as well on overall dermal thickness. In these experiments the skin was obtained from either the breast or the abdomen. The method used in these experiments to create the skin explants was the manual Watson knife, in which the thickness is set by adjusting the gap between the blade and the knife. The manual pressure applied by the user influences the dermal thickness to an even greater extent when compared to using an air driven dermatome.

 In our experiment we divided the skin explants into two groups based on varying dermal thickness measured with a digital micrometer, as the epidermal thickness from one sample was assumed to be constant throughout. We hypothesized that dermal thickness should not influence the migration of skin cells from the explants as this in theory should only occur from the cut edges and should not be influenced by dermal thickness. Therefore, we hypothesized that there should be similar amounts of migration when thicker and thinner samples were compared.

Finally, as a last strategy we tried to model our constructs on other aspects of the skin grafting technique that is practiced clinically. Once skin grafts are

secured onto a wound bed dressings are applied over the graft and are often secured with a tie over suture. This is all aimed at reducing the disruptive shear forces that occur between the graft and the wound bed which result in breakage of the fibrin bonds and graft failure. In our constructs we postulated that putting another layer of electrospun scaffold on top of the skin explants would act as a dressing and prevent the displacement of skin explants in the media. Given that we know the scaffolds support skin cell migration and are porous in nature, media would still be able to diffuse through the scaffolds to nourish the skin explants (Figure 6.1).
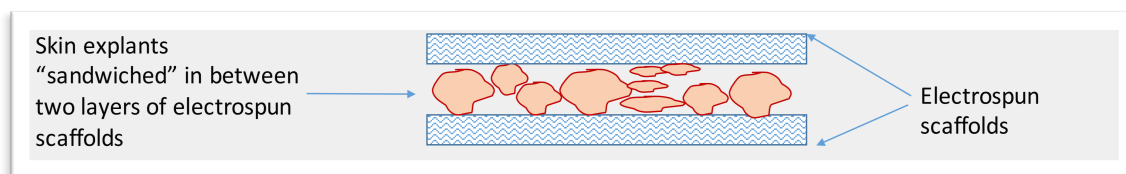
Figure 6.1: *Schematic illustrating skin explant between two layers of electrospun scaffolds*

6.3 The effect of varying the concentration of fibrin on cellular migration from skin explants.

These experiments aimed to assess the effect of varying the concentrations of the constituent components of fibrin on outward cellular migration from the skin explants. This was assessed quantitatively by using Rose Bengal Staining at 0, 7, and 14 days respectively to map the outgrowth of cells from the skin explants that were mixed with fibrin on tissue culture plastic. Furthermore, at 14 days we undertook DAPI staining to confirm that presence of skin cells from the cut edge of the skin explant pieces. Additionally, we repeated these experiments using trilayer electrospun scaffolds instead of tissue culture plastic, which enabled us to obtain H&E images of the explant-fibrin-scaffold interface.

Rose Bengal Staining

Rose Bengal is a derivative of fluorescein and stains by localizing primarily in cellular nuclei and, to a lesser degree, in other organelles(222). It can be used to quantify the amount of cellular outgrowth from skin explants without affecting their viability. This allows for a continuous *in vitro* analysis of the effect of varying the concentration of fibrinogen and thrombin on outward

cellular migration from the cut edges of skin explants. The various combinations of thrombin and fibrinogen used in the experiments are detailed in Table 11.

Experiment	Concentration of Thrombin (I.U) /ml	Concentration of Fibrinogen mg/ml		
A	2.5	18.75	67	86.5
B	4.5	18.75	67	86.5
C	6.5	18.75	67	86.5

Table 7: *Various concentrations of thrombin and fibrinogen used in constructs to test their effect on the cellular outgrowth from skin explants.*

For these experiments the split thickness skin explants were placed in the centre of each well in a six well plate. To this varying concentrations of thrombin and fibrinogen were added on top of the skin explants as outlined in table 1 that resulted in a fibrin clot. Rose Bengal was then added according to the methods outlined in chapter 2 and photographed as a day 0 record. Following this, the constructs were cultured in 10% serum Green's and stained at day 7 and 14. The total areas stained by the Rose Bengal at each of these time checkpoints were measured in mm^2 using ImageJ software (Figure 6.2).

There was a progressive increase from day 0 to day 14 in the Rose Bengal

stained area the thrombin groups 2.5 and 4.5 for all concentrations of

fibrinogen. Constructs with a thrombin concentration of 6.5, reflected a

decrease in the stained area from day 7 to day 14 at all fibrinogen

concentrations apart from 86.5mg.

Group Analysis

Thrombin 2.5

Constructs containing thrombin/fibrinogen 2.5mg/18.75mg showed

significantly greater stained areas at day 7 and day 14 compared to the other

two fibrinogen concentrations (p=0.0041 at day 7 and p= 0.0005 at day 14)

(Figure 6.2a).

Thrombin 4.5

Although constructs containing fibrinogen 86.5mg had a greater area stained

by Rose Bengal at day 14 this was not statistically significant compared to the

other two-fibrinogen concentrations (Figure 6.2b).

Thrombin at 6.5

These constructs had significant differences in the Rose Bengal area stained at day 7 and 14 (table 12). This group demonstrated that with a fibrinogen concentration of 18.75mg at day 7 there was significantly more outward Rose Bengal staining than the other fibrinogen concentrations at this time point. Furthermore, constructs with fibrinogen concentration of 86.5mg had significantly larger stained areas compared to the other fibrinogen (67mg and 18.75 mg respectively) concentrations at 14 days (Figure 6.2c).

A.

B.

Thrombin 4.5

C.
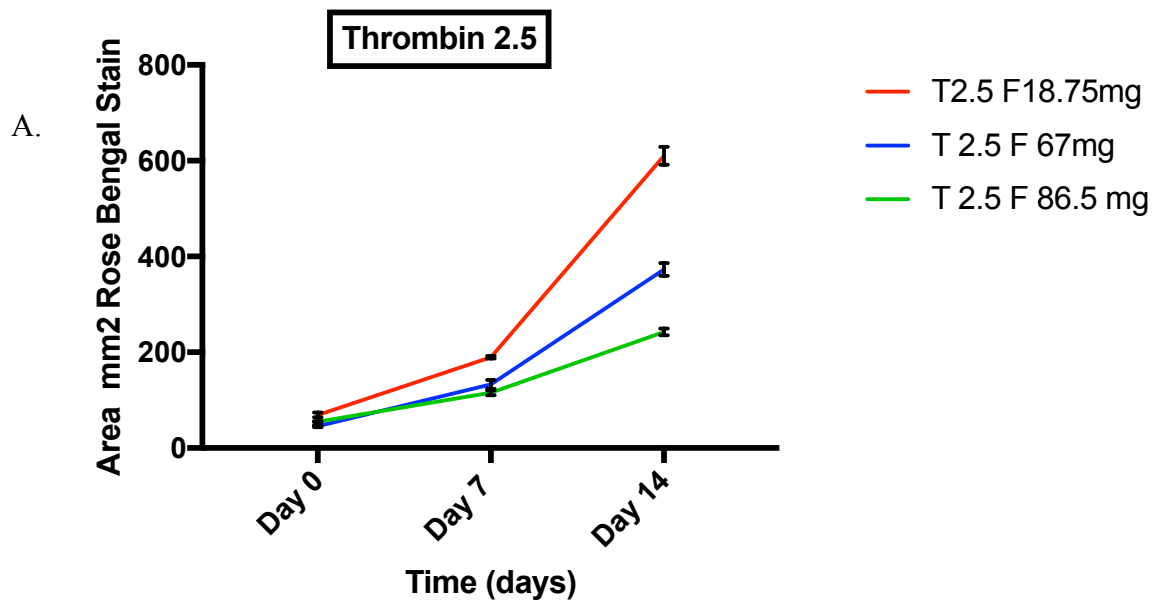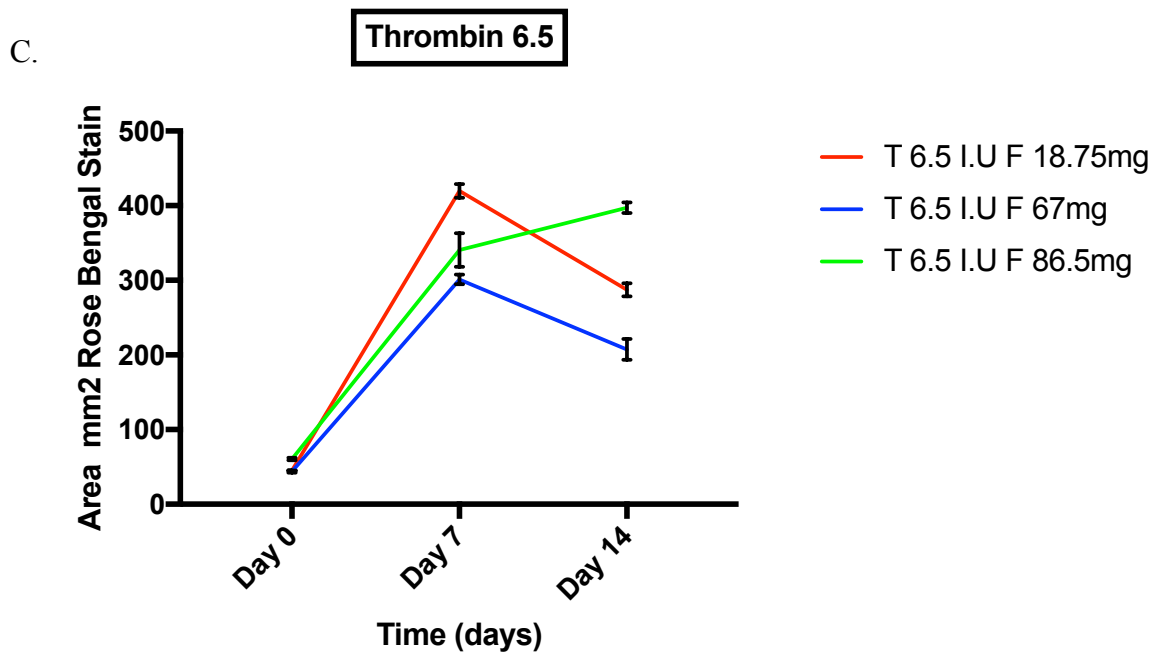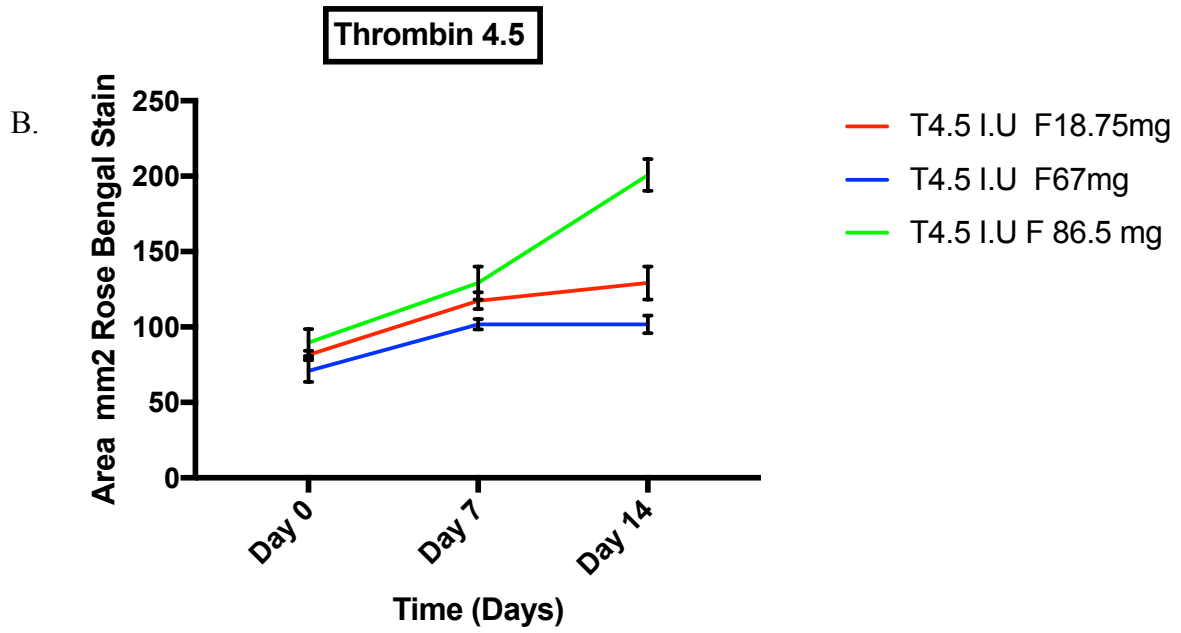
Thrombin 6.5

Figure 6.2: *Area of Rose Bengal Staining over a 14-day culture period using constructs containing various concentrations of thrombin and fibrinogen with skin explants. A: thrombin 2.5 I. U/ml; B: thrombin 4.5 I. U/ml; C: thrombin 6.5 I. U/ml. (Mean±SEM).*

DAPI Staining

This was undertaken for fixed constructs at day 14 to demonstrate cellular

migration from the cut edges of the skin explants when combined with varying

concentrations of thrombin and fibrinogen on tissue culture plastic. The results

indicate that cellular migration surrounding the skin explants were the most for

the thrombin 2.5 groups when all concentrations of fibrinogen were evaluated.

The migration of skin cells from the cut edges of skin explants seemed to

decrease as the fibrinogen concentration increased. There was negligible to

absent migration of skin cells from the skin explants in the other

thrombin/fibrinogen group combinations as demonstrated in Figure 6.3 apart

from thrombin/fibrinogen 4.5/86.5 which clearly illustrates cellular migration

from the edge of the skin explant on tissue culture plastic.
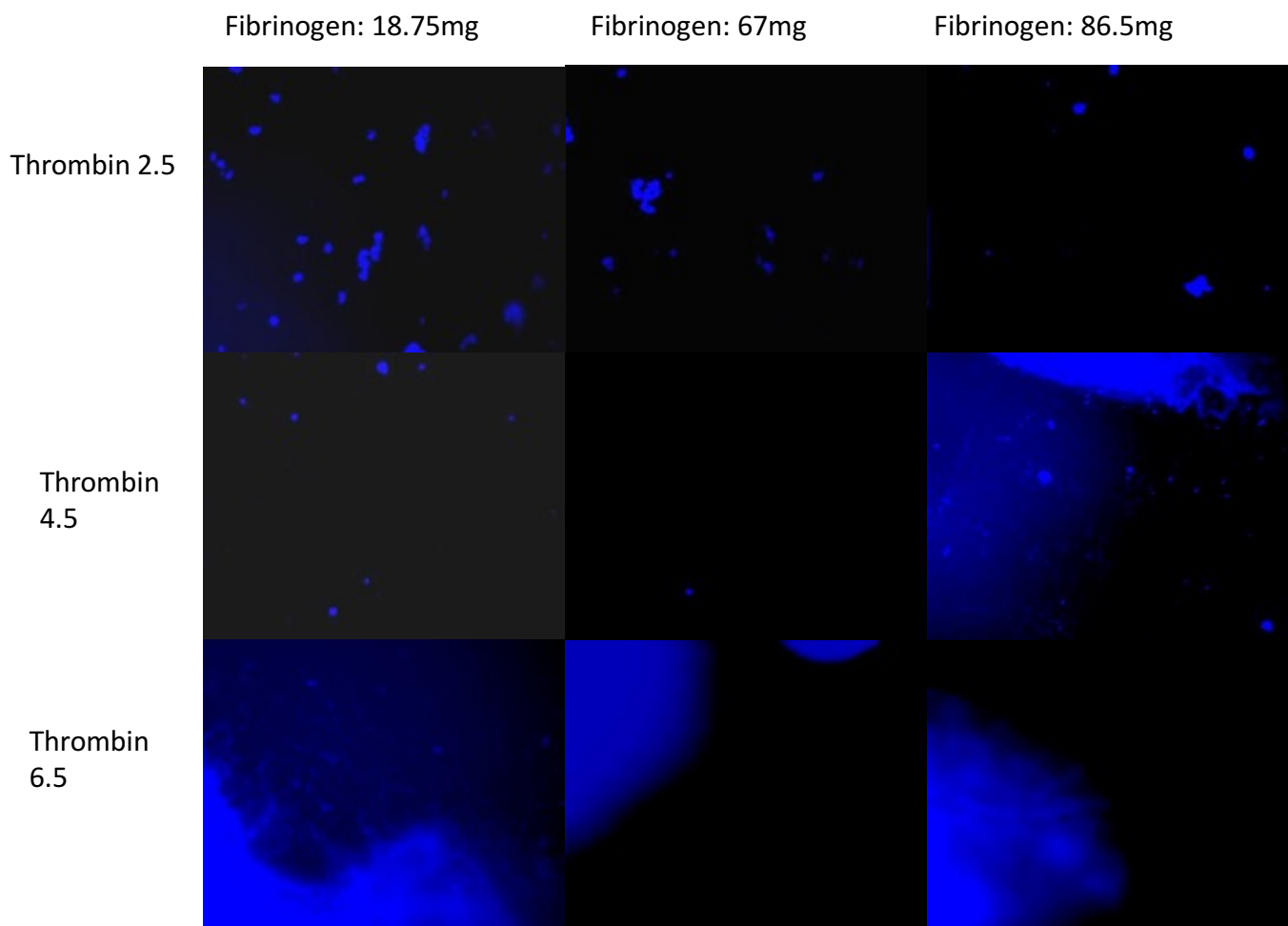
Figure 6.3: *DAPI fluorescence staining of varying concentrations of thrombin and fibrinogen combined with skin explants on tissue culture plastic.*

Hematoxylin and Eosin Staining.

These were undertaken on fixed constructs on day 14. Skin explants were cultured on templated trilayer PHBV/PGLA 75: 25 scaffolds with varying concentrations of fibrin constituents for 14 days as noted in Table 1. These visually highlight the electrospun scaffold/skin explant/fibrin architectures and evidence of integration, if any between the two. Figure 6.4 illustrates that the

skin explants were closely applied to the scaffolds in the thrombin 2.5 group, at all concentrations of fibrinogen. Increasing the concentration of thrombin to 4.5 and 6.5 results in a significant gap between the skin explants and the electrospun scaffolds. This was mostly obvious at fibrinogen concentrations of 86.5mg/ml. There were very little visible cellular structures present on the electrospun scaffolds that migrated from the cut edge of the skin apart from some material just adjacent to the skin explants in the thrombin 2.5 group.

| | Fibrinogen 18.75mg | Fibrinogen 67 mg | Fibrinogen 86.5 mg |

Thrombin 2.5

Thrombin 4.5
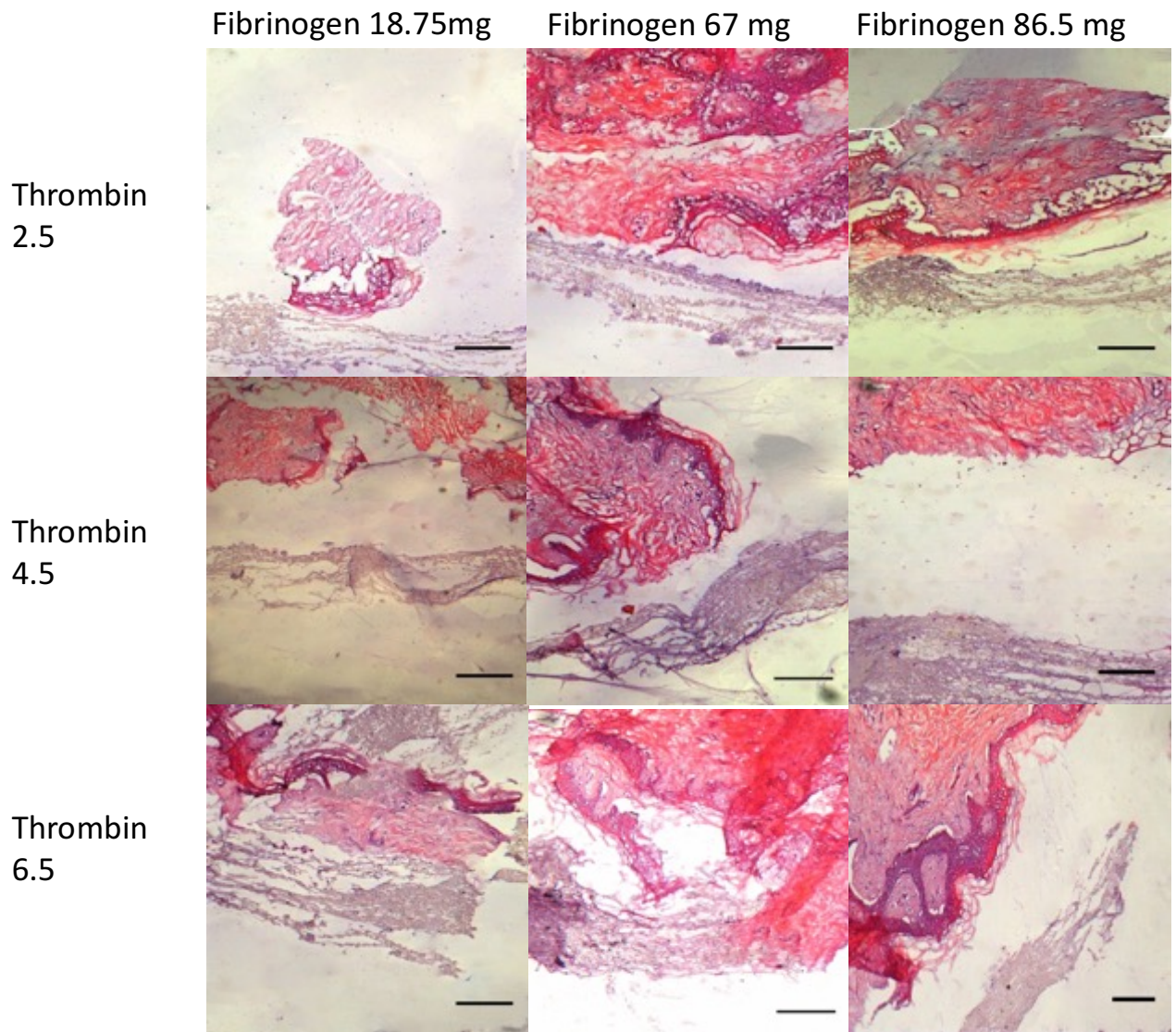
Thrombin 6.5

Figure 6.4: *Representative light microscopy H&E stained sections of skin explants seeded on templated trilayer electrospun scaffolds with various concentrations of fibrinogen and thrombin cultured over 14 days.   Scale bars of 0.2 mm (100X magnification).*

6.4 Investigating the effect Tisseel on skin explant handling and cellular migration along fibres of trilayer electrospun scaffolds

These experiments aimed to assess the ability to use Tisseel, a commercially available fibrin sealant that is already in clinical use, to fix skin explants onto electrospun scaffolds. We also wanted to assess if there was any difference, if any when Tisseel was used with templated versus non-templated PHBV/PGLA (75:25) electrospun scaffolds. Constructs containing trilayer templated and non-templated electrospun scaffolds were cultured with skin explants mixed with Tisseel. They were cultured for 7,14 and 21 days respectively.

AlamarBlue^{TM} assay

Absorbance for skin explants and Tisseel cultured on trilayer templated scaffolds and non-templated scaffolds were read and the absorbance for scaffolds of each type with no skin explants and Tisseel were subtracted from these values. The absolute values are represented at days 7, 14, and 21. It was demonstrated that the skin explants with Tisseel were viable throughout the 21-day testing period on both templated and non-templated scaffolds (Figure 6.5).

Cellular metabolic activity in constructs were similar to those without Tisseel where a decrease was noted from day 7 to day 14 followed by an increase in

metabolic activity steadily through today 21. There was no significant difference in metabolic activity when skin explants with Tisseel were seeded on templated versus non-templated electrospun scaffolds (p=0.7928).

.
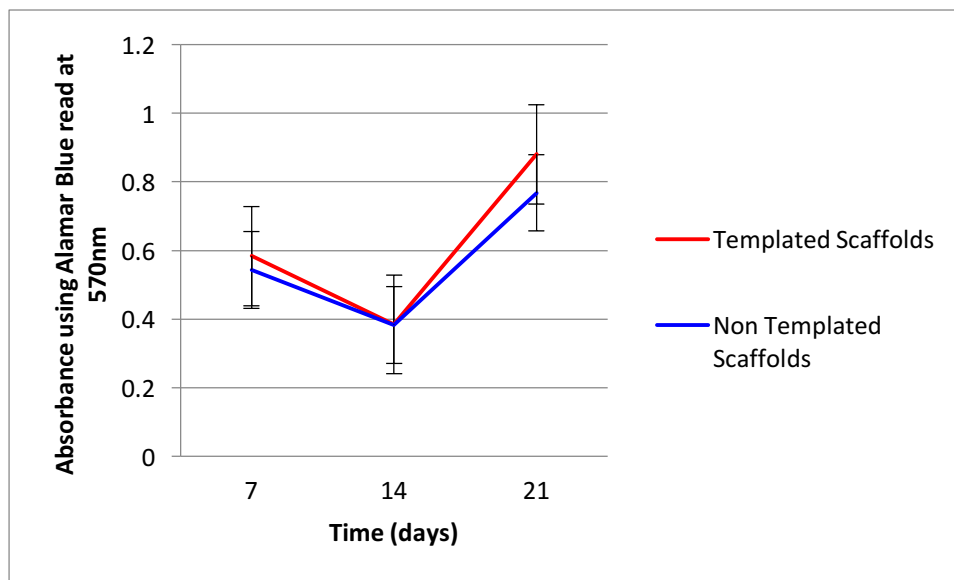
Figure 6.5 *Metabolic activities of skin explants with Tisseel on templated and non-templated trilayer electrospun scaffolds over a 21-day experimental period as determined by absorbance of AlamarBlue stain (Mean±SEM).*

DAPI Staining

This was undertaken for constructs fixed at day 7, 14, and 21 to determine if cells from the cut edges of the skin explants were now able to migrate along the fibres of the electrospun scaffolds in the presence of Tisseel. We also wanted to assess if there were any differences between templated and non-templated scaffolds. Cells were seen to be leaving the surrounding area around

the skin explant pieces that progressively increased over time and was most

visible on day 21. This level of cellular migration was much more than that

observed when skin explants were simply placed on scaffolds with no Tisseel.

However, when compared to tissue engineered skin constructs using DED at

the same time checkpoints as a positive control, there was not as much cellular

migration and proliferation observed even in the presence of Tisseel.

Non-Templated scaffolds   Templated scaffolds

Day 7

Day 14

Day 21
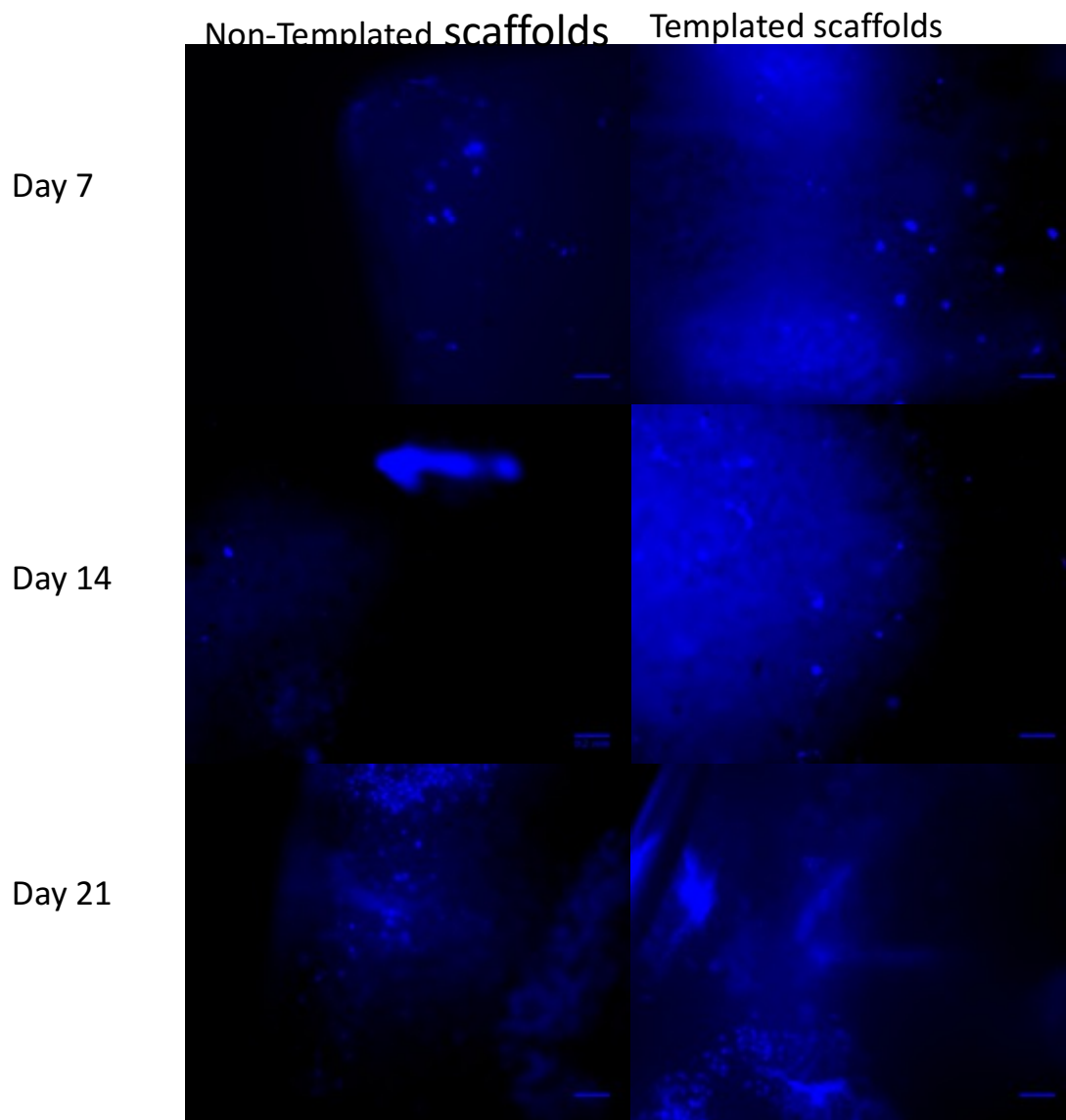
Figure 6.6: *DAPI staining of constructs consisting of non-templated scaffolds and templated scaffolds cultured with skin explants and Tisseel. Scale bar=0.2mm*

Haematoxylin and Eosin Staining.

This was undertaken on fixed constructs at day 7, 14, and day 21 to demonstrate consecutive skin explant/Tisseel/scaffold architectures. Frozen sections of constructs stained with H&E demonstrated some cellular outgrowth from the cut edges of the skin explants onto the fibres of the electrospun scaffolds. There was more integration of the skin explants with the scaffolds in the presence of Tisseel compared to skin explants cultured without Tisseel. This however did not hold for non-templated scaffolds cultured with skin explants on day 21, as the Tisseel seemed to have set in between the skin explants and the scaffold creating a dense clot between the skin cells and the scaffold fibres (Figure 6.6).
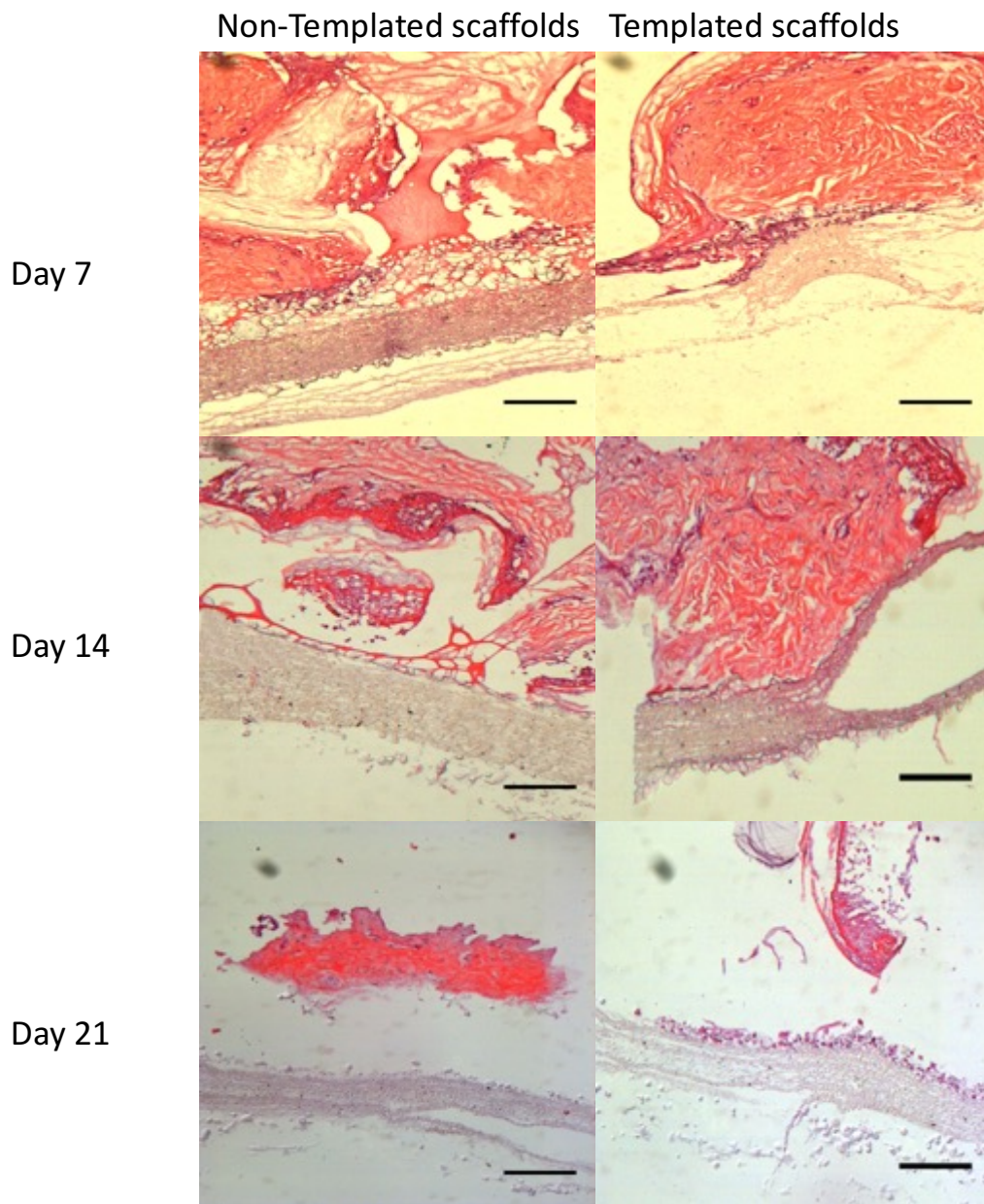
Figure 6.7: *Representative light microscopy H&E stained sections of skin explants seeded on templated and non-templated trilayer electrospun scaffolds with Tisseel and cultured over 14 days. Scale bars of 0.2 mm (100X magnification).*

Sirius Red Assay

At day 7, 14, and 21 Sirius red assays were undertaken to estimate the total collagen produced overtime by the skin explants seeded on trilayer electrospun scaffolds with Tisseel (Figure 6.7). The aim was to compare the amount of collagen deposited by proliferating fibroblasts from the skin explants compared to those from tissue-engineered skin using DED as a positive control. Furthermore, we aimed to compare Tisseel/skin explant constructs to those without Tisseel. There was no significant difference in total collagen produced per gram of scaffold when Tisseel was used between templated and non-templated electrospun scaffolds. However, comparing these skin explant/Tisseel constructs to DED there were significant differences in collagen produced with the latter being significantly more at day 7 compared to non-templated scaffolds with Tisseel and at day 14 for all constructs containing Tisseel (Figure 6.8). However, at day 21, although total collagen produced per gram was more in the DED samples, this was not significantly different to those constructs containing Tisseel (Table 13). Furthermore, templated constructs containing skin explants/ Tisseel had significantly more collagen produced per gram compared to those constructs containing skin explants alone at day 14.

A
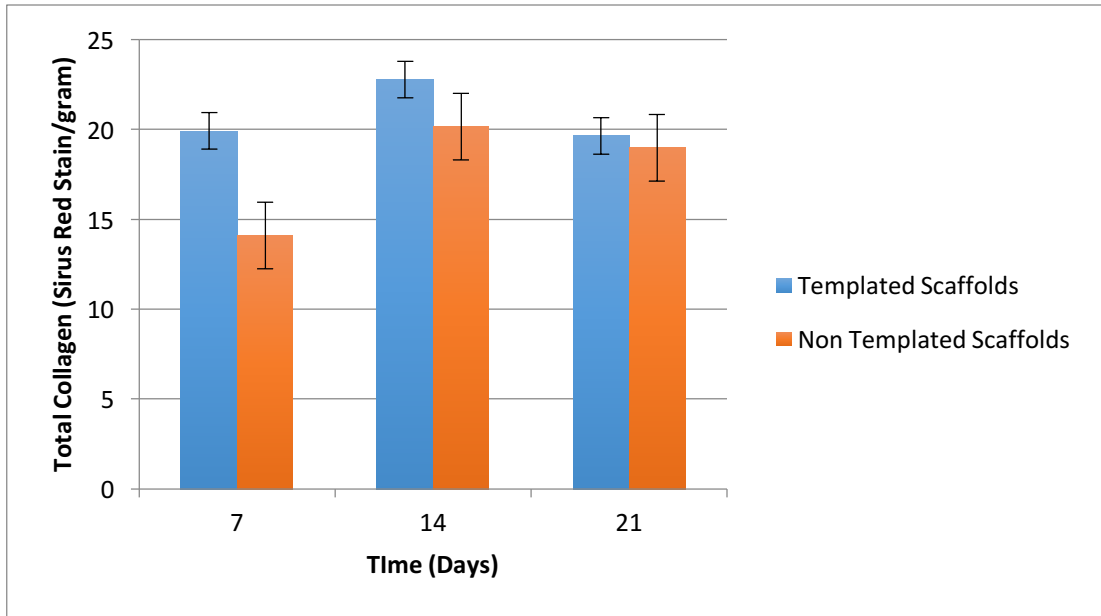
B.
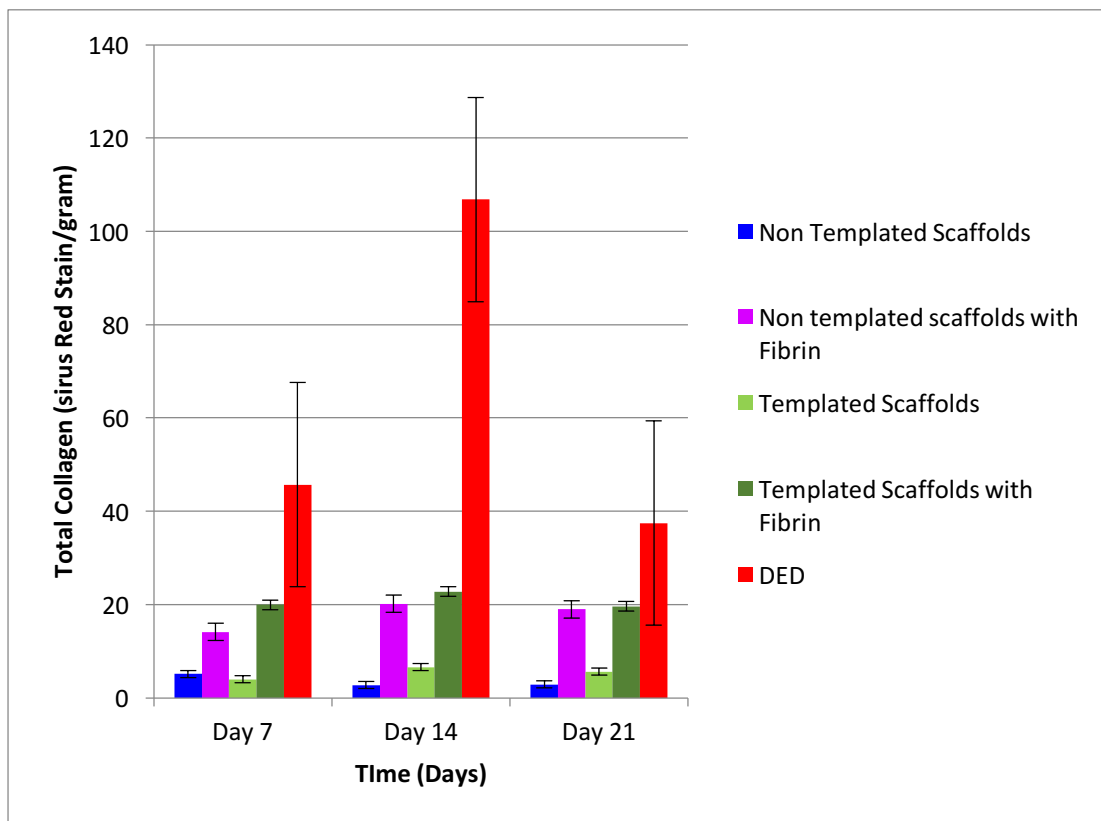
Figure 6.8: *Sirius red staining after 7, 14, and 21 days comparing a: non-templated and templated with skin explants and Tisseel; b: skin explants on scaffolds, skin explants with Tisseel and DED. (N=3±SEM).*

Construct A	Construct B	P Value
Non templated scaffolds with Tisseel Day 7	DED Day 7	0.0255
Non templated scaffolds with Tisseel Day 14	DED Day 14	0.0055
Templated scaffolds with Tisseel Day 14	DED Day 14	0.0025
Templated scaffolds with explants no Tisseel	Templated scaffolds with Tisseel Day 14	0.0104

Table 8: *Illustrating significant P values comparing Sirius Red Staining of various constructs using Unpaired Student's T test.*

Section Discussion

The aim of these experiments was to determine if a clinically used sealant like Tisseel would be suitable to adhere the skin explants onto the electrospun scaffolds and at the same time facilitate cellular migration. Tisseel is widely used in hospitals across many specialties in the United Kingdom. In plastic surgery, it is used mainly as a haemostat when there are no identifiable vessels

to be sutured or coagulated on a bleeding wound bed. As Tisseel is marketed mainly as a haemostat and a sealant, the concentration of thrombin is very high which facilitates prompt coagulation, but also a fibrin clot with thin tightly packed fibrin strands.

The experiments in the previous section tested fibrin clots produced by low thrombin concentrations which usually are found in adhesives. These products are used infrequently in the hospital environment to adhere skin grafts or flaps to wound beds as it is more expensive than suturing. Therefore, we wanted to assess if Tisseel, which is already part of theatre stock lists, would facilitate cellular migration from skin explants.

Adding Tisseel to constructs did not adversely affect the viability of the cells as their metabolic activity increased progressively throughout the experimental period. Qualitative analysis using DAPI showed that there was more cellular migration from skin explants in constructs containing Tisseel compared to skin explants cultured without Tisseel. Furthermore, when assessing total collagen produced, it was found that constructs containing Tisseel showed more collagen production than those without, which was significant at the 14-day culture period for templated constructs. Although DED constructs had

significantly higher amounts of collagen at day 7 and day 14 compared to Tisseel constructs, by 21 days this difference became insignificant. This suggests that over time, the fibroblasts from the skin explants that are adherent to the scaffold with the help of Tisseel, were able to proliferate and start producing collagen. This is one of the key steps in wound healing as proliferating fibroblasts produce signals to stimulate the further migration and proliferation of keratinocytes.

There were however a few challenges encountered when handling Tisseel. The most significant one is that it sets quickly; as the jet of thrombin and fibrinogen exits the syringe it forms a ball of fibrin that is quite tough. There was about 5-6 seconds to manipulate skin explants onto the scaffold before the fibrin began to set into a solid clot. Furthermore, because it was a very dense clot, some of explants were bunched together rather than being flat onto the surface of the electrospun scaffold which is needed for outward cellular migration onto the scaffolds. We were able to achieve correct positioning of the skin explants on the scaffolds when thrombin/fibrinogen concentrations of 2.5 I. U/18.75 mg were used and like the Tisseel clot was stable (still structurally visible with the skin explants present) throughout the 21-day experimental period.

6.5 Investigating the effect of varying dermal thickness on cellular migration from the cut edges of skin explants.

The aim of these experiments was to investigate the effect of varying dermal thickness on cellular migration from the cut edge of skin explants. It aimed to determine if thinner (with respect to the dermis) skin explants had more outward cellular migration versus samples containing thicker dermis. There is no consensus on a thin vs a thick skin explant. As a result, we used the thinnest setting on the Watson knife for the thin explants which were all less than 0.3mm and the thickest setting on the Watson knife for a thick skin explant – all > 0.7mm. Split thickness skin of varying dermal thickness were categorized into two groups: thick and thin after measuring each square piece of skin explant with a digital micrometer. As all samples in the experiment came from the same source skin, it was assumed that the epidermis was of a constant thickness and the variability in the readings resulted from the varying amounts of dermis present. Squares of skin explants were then minced into fine pieces and placed in the centre of a six well plate. They were cultured for a period of 7days. The dermal thicknesses of the 2 groups are summarized in Table 14 below.

Group A: thin dermis /mm	0.282	0.220	0.154	0.256
Group B: thick dermis/mm	2.031	1.030	1.761	0.716

Table 9 *Various thickness of skin explants used in experiments (mm).*

Rose Bengal Staining of skin explants of varying dermal thickness.

This stain was able to evaluate ongoing cellular migration from the skin explants outwards from day 0 to day 7 (Figure 6.9). Figure 6.9A shows the area occupied by the stain after a few hours of being in culture as a baseline. The blue bars indicate the explants that belonged to the samples with thin dermis and the red bars those with thick dermis. At this point, the area of stain surrounding the explants in both the groups were very similar as equal squares of explants were used with the same amount of rose bengal staining. The Rose Bengal stain was removed and the explants were returned to culture for 7 days. At day 7 (Figure 6.9B) there is a significant increase in the stained area surrounding the skin explants with thin dermis compared to those with thicker

dermis (p= 0.0026 Kruk shall Wallis test for multiple groups assuming non parametric distribution)

This is better illustrated in Figure 6.9C where the mean of each replicate value of the area of rose bengal stain per skin explant has been plotted at day 0 and day 7 respectively. This clearly illustrates that the explants with thinner dermis, overtime results in more outward cellular migration of skin cells onto scaffolds versus thicker skin explants.

A.

B.

C
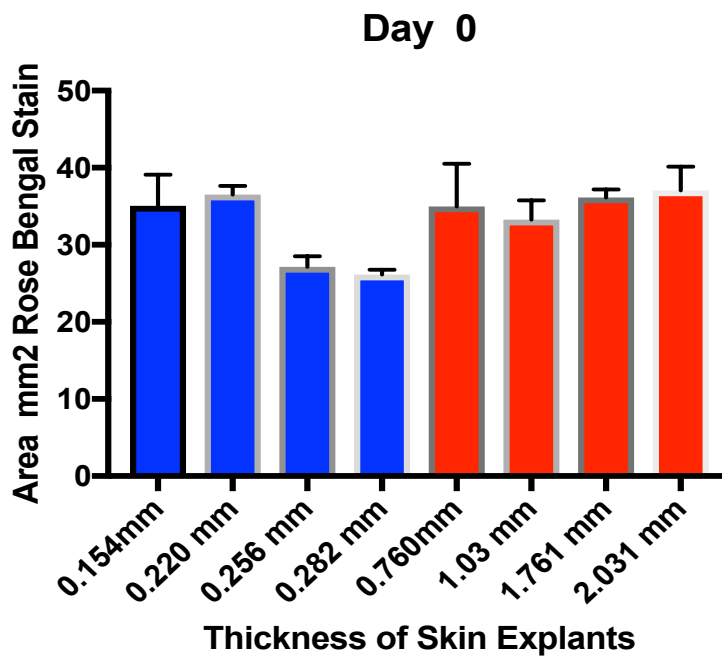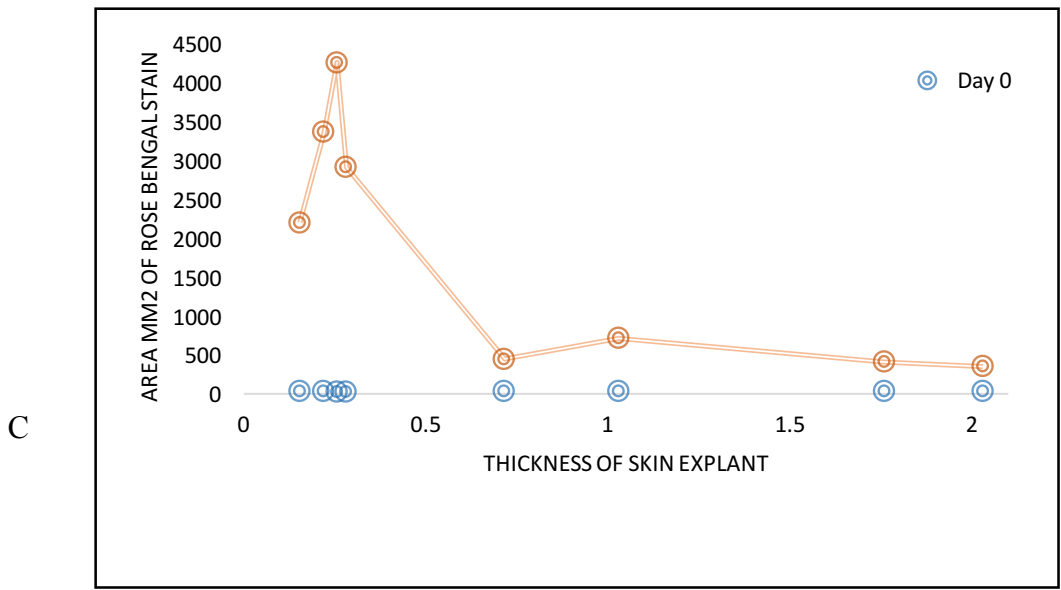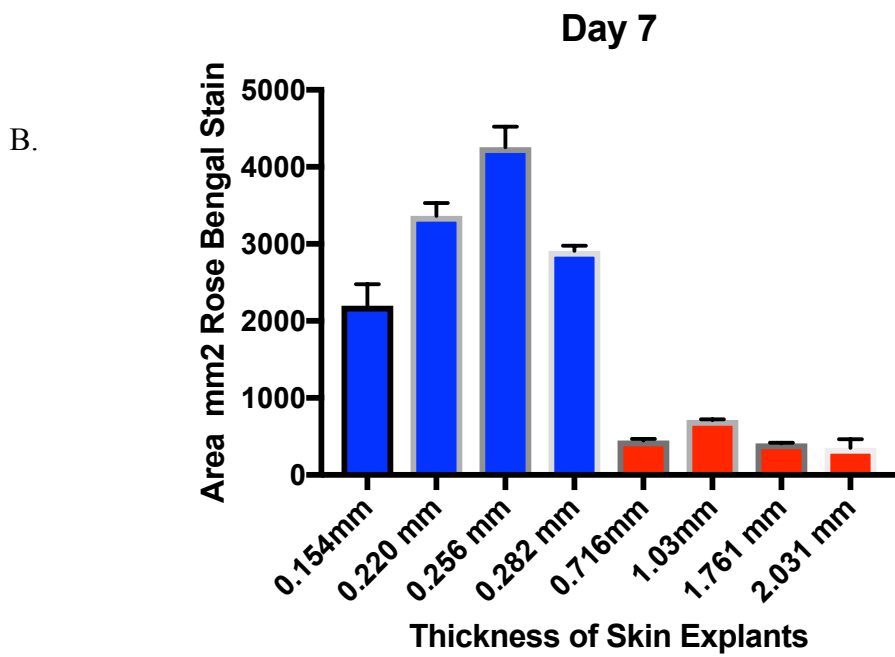
Figure 6.9: *Rose Bengal stained area in mm2 illustrating cellular migration from skin explants. A: Skin both thick and thin at day 0, B: Skin explants at day 7; C: The mean of the area staining per explant thickness at day 0 and day 7.*

Section Discussion

The aim of this work was not only to produce a biodegradable membrane, but also to develop a technique whereby skin explants from the patient would be used as a cell source to populate the fibres of the scaffold in a reproducible manner. In that regard, we needed to scrutinize the properties of the skin explants both in terms of thickness and placement onto the electrospun scaffold to assess if any of these factors influenced outward cellular migration. Many groups have published tissue-engineered models using isolated cultured autologous cells as skin substitutes(223). However as discussed in the introduction these have not made a sustained impact on the reconstructive options available to surgeons and hence patients. The advantage of using skin explants is that they contain fibroblasts, which serve to stimulate the migration and proliferation of keratinocytes. We have shown that our scaffolds facilitate the migration and proliferation of both these cells. Therefore, the idea here is to use skin explants in one step, as a cell source to populate the fibres of the electrospun scaffolds.

In order to translate a technique clinically, it has to be easily reproducible to reduce the effect of patient variability on a successful outcome. The less precise the requirements for the technique, the more chance of it becoming

successful in the clinical environment due to the unavoidable influence of human error and patient physiology. One of the oldest and most common skin reconstructive techniques used in clinical practice is the skin graft. It is a reliable method of reconstruction as the technique is easily reproducible. There are no recommended dermal thickness ranges for skin grafts apart from clinical indications – for example in burn contracture reconstruction full thickness grafts are preferred as they have less secondary contraction compared to split thickness skin grafts. The dermal thickness often influences the method of fixation used for the graft on the wound bed. Split thickness grafts with less dermis have less primary contracture (elastic recoil at the time of harvesting due to the amount of elastin in the dermis) and as a result they are able to lay flat on a wound bed without having to suture the edge. Tissue glue can be used and a tie over dressing. This is not the case for full thickness grafts, where there is significantly more primary contracture. The piece of skin tends to therefore curl in and not lay flat on the wound bed compared to grafts with thinner dermis.

In these experiments a Watson knife was used to harvest the skin into grafts and by adjusting the blade we were able to obtain skin samples containing variable amounts of dermis. This explains the variation in the thickness of skin explants that were obtained in each group. The Watson knife is not commonly

used to harvest skin grafts as a matter of routine as it has been superseded by the air driven dermatome, which results in grafts with a more consistent dermal thickness. Our data supports the use of skin explants with less dermis for our proposed technique as there was a significant increase in the stained area around the explants with less dermis compared to those with more dermis. This reflects increased cellular migration from the cut edges of the skin explants. Furthermore, thinner explants handle easier than thicker ones, due to less elastic recoil which results in more contact between the fibres of the scaffold and the overlying cells, increasing their chances of migrating out of the explant and onto the scaffold. Furthermore, previous unpublished work from our group demonstrated that the skin explants did not need to be orientated specifically on the underlying scaffold as skin cells tend to migrate from the cut edge of the explants as long as the skin explant is in contact with the fibres of the electrospun scaffold. Therefore, increasing the surface area of these edges by mincing the squares of skin facilitates increased contact with fibres of the scaffold and consequently increased cellular migration throughout the scaffold.

6.6 The sandwich technique to increase cellular migration from the cut edges of skin explants.

The aim of these experiments was to determine whether the placement of the skin explants between two layers of electrospun scaffold would aid cellular migration along the fibres of the scaffold onto which the explants were placed. Skin explants were placed in the centre of a 1.5 cm x 1.5 cm square piece of electrospun scaffold. Onto this, a further 1.5 x 1.5 cm square piece of scaffold was placed on top of the skin explants in order to keep them in place. Constructs were then raised to an air liquid interface and cultured for 7, 14, and 21 days.

AlamarBlue assay

Absorbance for skin explants cultured between two layers of electrospun scaffold, both templated and non-templated were read, the absorbance obtained from control scaffolds with no skin explants was subtracted from these values. The absolute values are represented from days 7, 14, and 21. It was demonstrated that the skin explants cultured between two layers of electrospun scaffold were viable throughout the 21-day testing period for both templated and non-templated scaffolds (Figure 6.10)

Cellular metabolic activity in constructs similar to those of skin explants cultured on a single layer of electrospun scaffold increased from day 7 through today 21. There were no significant differences in metabolic activity when comparing templated and non-templated at any of the time checkpoints of day 7 (p=0.3471), day 14 (p=0.7995) and day 21 (p=0.3391).
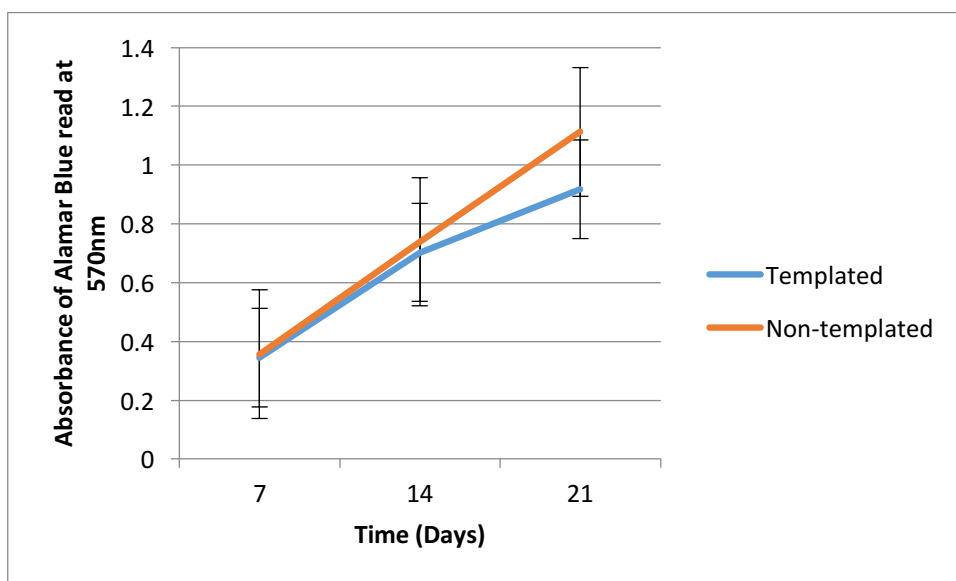
Figure 6.10: *Metabolic activities of skin explants seeded between two layers of electrospun scaffolds over a 21-day period as determined by absorbance of AlamarBlue stain (Mean±SEM).*

Haematoxylin and Eosin Staining.

This was undertaken on constructs fixed in formaldehyde at day 7, 14, and 21 to demonstrate consecutive skin explant/scaffold architectures. Frozen sections of constructs containing skin explants between two layers of electrospun scaffolds both templated and non-templated were stained with H&E. The images clearly demonstrate the position of the skin explants between the two layers of electrospun scaffolds. There was evidence of some cellular material on the scaffolds, which increased especially between the 14 to 21-day culture period (Figure 6.11)

	Non-Templated Scaffold	Scaffold
Day7		
Day 14		
Day 21		
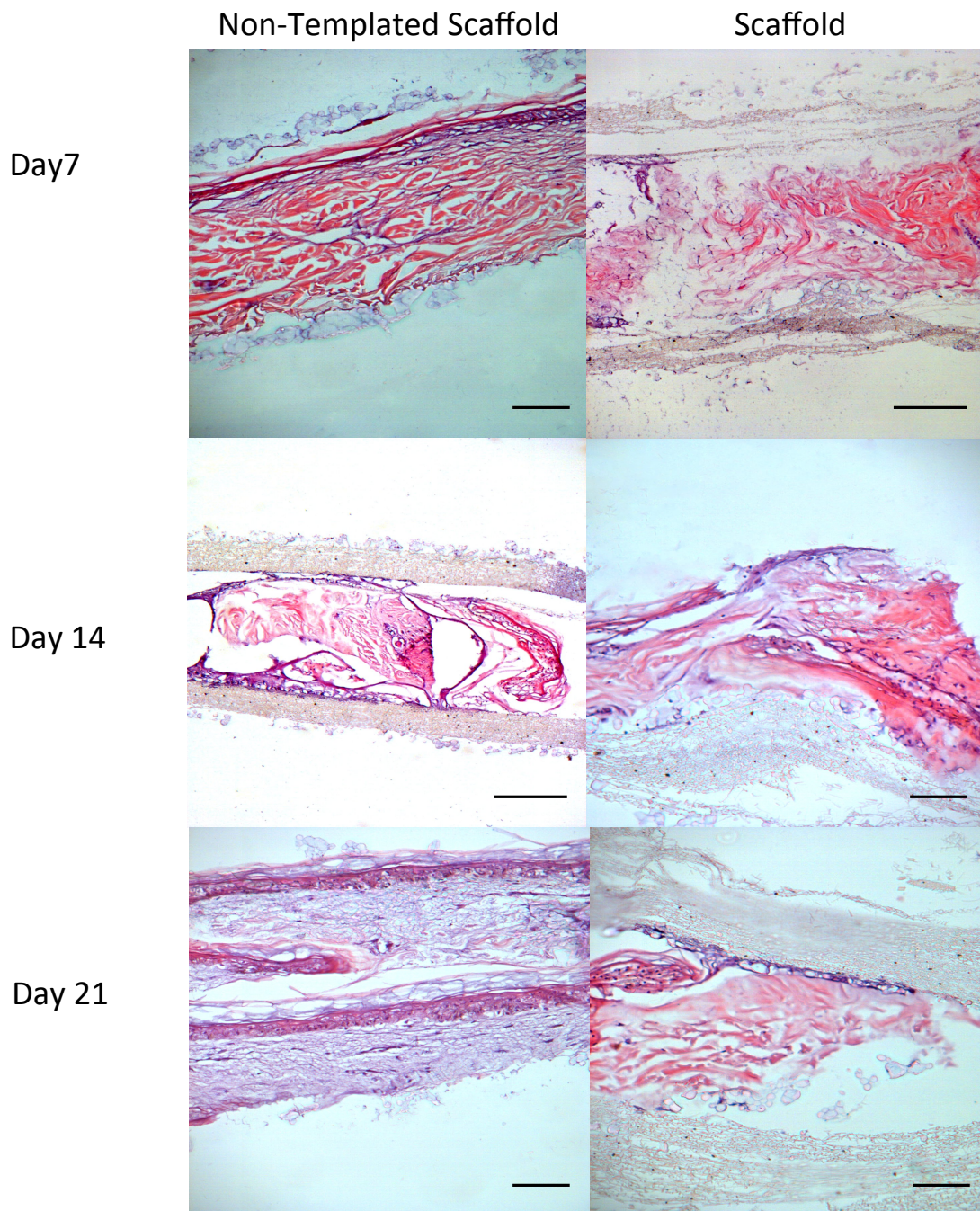
Figure 6.11: *Representative light microscopy H&E stained sections of skin explants seeded in between two layers of electrospun scaffold both templated and non-templated. Scale bars of 0.2 mm (100X magnification).*

Sirius Red Assay

At day 7, 14, and 21 Sirius red assays were undertaken to estimate the total collagen produced overtime by the skin explants placed in between the two layers of trilayer electrospun scaffolds (Figure 6.12). The aim was to quantify and compare the amount of collagen deposited by proliferating fibroblasts in electrospun scaffold sandwich from the skin explants to constructs containing tissue engineered skin using DED as a positive control. Furthermore, we aimed to compare skin explant/bilayer electrospun scaffold constructs to those cultured on monolayers of electrospun scaffold. There were no significant differences in total collagen produced per gram of scaffold when two layers of electrospun scaffold were used when comparing templated and non-templated scaffolds (Figure 6.12a). However, comparing these skin explant/bilayer constructs to DED tissue engineered skin there were significant differences in collagen produced with the latter being significantly more at 7 and 14 days (Figure 6.12b) (table 6). Interestingly at day 21 although in absolute values the amount of collagen produced by the DED constructs were more, there were not significantly different from templated and non-templated bilayer constructs respectively (p= 0.1003; p= 0.2288)

Furthermore, although skin explants/ bilayer constructs had more collagen

produced per gram compared to those constructs containing skin explants

alone in absolute terms this was only significant at day 21 (table 15).
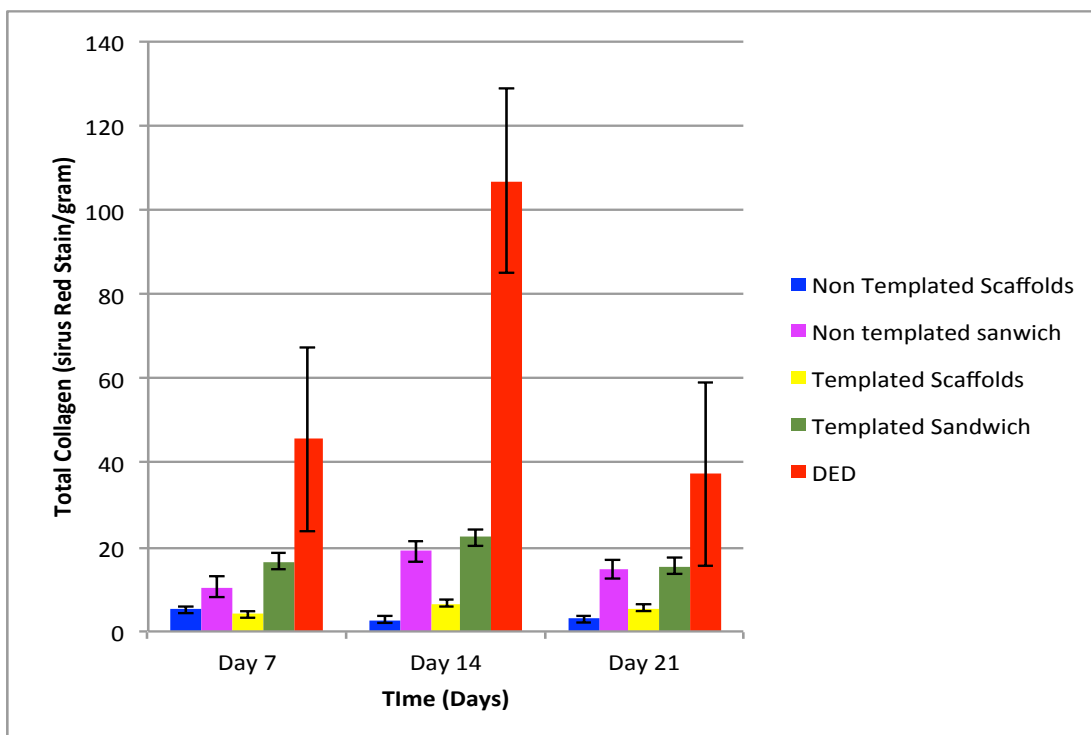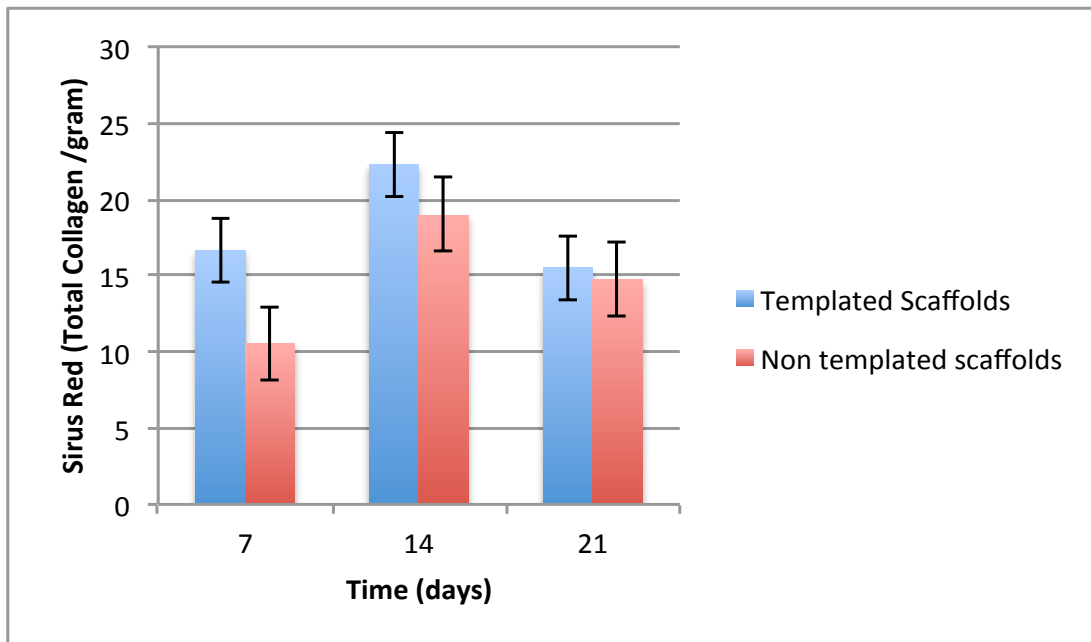
Figure 6.12: *Sirius red staining after 7, 14, and 21 days comparing a: non-templated and templated scaffolds used to create a sandwich construct with skin explants in the centre; b: skin explants on scaffolds, skin explants in between 2 layers of scaffolds and DED. (N=3±SEM).*

Construct A	Construct B	P Value
Non templated bilayer Day 7	DED Day 7	0.0078
Templated bilayer Day 7	DED Day 7	0.0256
Non templated bilayer day 14	DED Day 14	0.0037
Templated bilayer day 14	DED Day 14	0.0051
Non templated skin explants D 21	Non Templated bilayer Day 21	0.0108
Templated skin explants D21	Non Templated bilayer Day 21	0.0170

Table 10: *illustrating significant P values comparing various constructs from Sirius Red Staining using the Unpaired Student's T test.*

Section Discussion

This experiment aimed to optimize the migration of skin cells from the skin explants onto scaffolds by adding another layer of electrospun scaffold to act as a dressing on top of the skin explants. We felt this was an easy, reproducible technique to promote retention of the skin explants during media changes and *in vitro* AlamarBlue testing. Furthermore, placing scaffolds on top of the skin explants would not limit diffusion of nutrients and oxygen from media as it is porous and facilitates free movement of these molecules.

This idea is simply mimicking what would actually happen if this technique were to be transferred to an *in vivo* model. Once the skin explants are laid onto the scaffold, there would be a requirement for some dressing or material to protect it during the course of the experiments from shear forces. This would be the case even if fibrin were to be used. Clinically even if grafts are sutured or glued onto a wound bed, a layered compressive dressing is placed over the graft. This serves a number of purposes. Firstly, it reduces the amount of shear force that is transmitted to the graft during movement. It is also acts to absorb blood and tissue fluid from the wound bed to prevent it from accumulating between it and the graft, which would prevent direct graft to wound bed contact and thereby graft take. The results have indicated overall that these constructs were viable, and had more proliferating fibroblasts producing more

collagen than skin explant/scaffold constructs without the additional layer of scaffold on the top. Although there was not a lot of cellular migration onto the scaffolds at the start of the experiments, this progressively increased to day 21. This suggests that given time, cells contained in the skin explants would migrate out onto the scaffold and populate the fibres.

6.7 Chapter Discussion

The aim of this chapter was to optimize the migration of skin cells from the cut edges of skin explants onto the electrospun scaffolds. Previous chapters have demonstrated that given time, cells will migrate and populate the scaffold, however in the context of wound healing this should usually occur in approximately two weeks. A product that has a primary aim of augmenting wound healing must achieve this in a timely fashion. Skin and soft tissue cover of a wound is one of the fundamental aspects of plastic surgery, and most techniques revolve around trying to achieve this in a safe and effective manner. It significantly improves the patient's quality of life, as having to continuously dress a wound left to heal by secondary intention requires time, expertise, and a limitation of activities of daily living. In reality, wounds left open to heal by secondary intention take anywhere from 6 weeks to 3 months to achieve re-epithelization, and at times even longer depending on the

underlying pathology and patient factors. Furthermore, a break in the skin's

barrier albeit large or small always carries the risk of infection. Therefore,

although culturing skin explants on electrospun scaffolds result in eventual

cellular migration, we are observing that this starts most definitively from the

14- 21-day mark. Thereafter the cells would then need more time to populate

the scaffold and start proliferating to produce an extracellular matrix.

Therefore, we identified areas of improvement that could refine our technique

of using skin explants combined with trilayer templated electrospun scaffolds.

The first being our construct and the second introducing an *in vitro* wound

bed. This chapter focused on modifications of our technique by capturing

some aspects of clinical practice.

Fibrin was shown to be an appropriate adhesive for the skin explants. The

structure of the fibrin clot at low thrombin concentrations facilitates cellular

migration and movement through the clot structure (Figure 6.13).

Furthermore, lower concentrations of thrombin allowed for some

manipulation of skin explants. Although Tisseel is a commonly used product in

the clinical area as a sealant and haemostat, the thrombin concentration

required for this function maybe too high for our application -although over

time, cells may eventually migrate, the aim of the fibrin application is to

accelerate this. Nevertheless there are other commercially available FDA

approved fibrin products that can be used for this purpose(224). As a result,

we have chosen to use a thrombin/fibrinogen concentration of 2.5/18.75mg as

our fibrin constituents for the purpose of our experiments in the forthcoming

chapters.
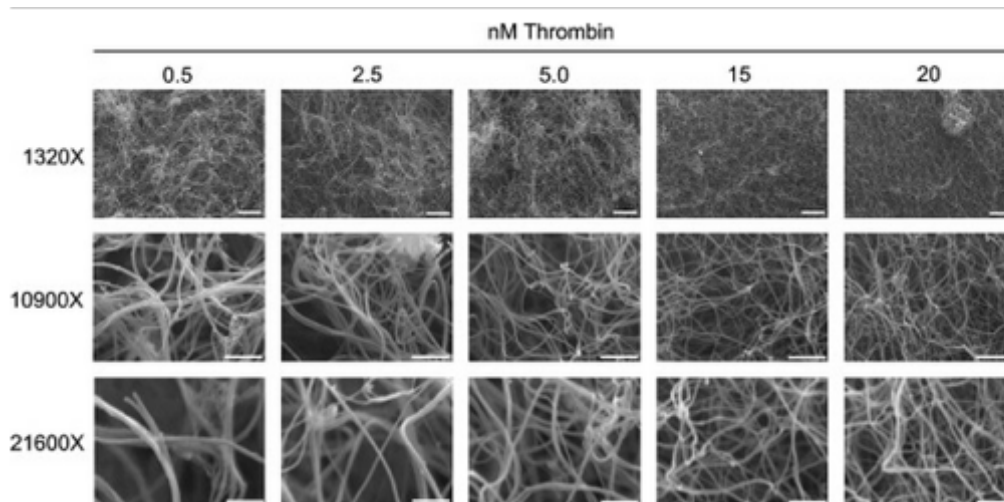
Figure 6.13: *The thrombin concentration present at the time of gelation dictates the fibrin clot structure. Scanning electron micrographs of fibrin clots formed by adding thrombin (0.5–20 nM) to solutions of purified fibrinogen (2 mg/mL). The scale bars indicate 10 μm (top row), 2 μm (middle row), and 1 μm (bottom row). Reproduced with permission from Elsevier (218).*

Furthermore, we were able to demonstrate that skin explants with less dermis

resulted in significantly increased cellular migration onto the scaffolds

compared to those with more dermis. This is an important finding as we are

able to define the characteristics of the skin explants that are required for the

technique to have the best chance of success. Additionally, clinically skin

explants with less dermis is a preferred option especially in patients with few

donor areas.

Dermis retention in wounds is essential for wound healing and donor

regeneration. Keratinocyte stem cells live in the bulge area of the hair follicle,

and migrate upwards when stimulated to form basal keratinocytes and

eventually re-epithelization the skin. These hair follicles are located within the

reticular dermis and ensuring that this part remains on the patient facilitates

eventual wound healing of donor sites(225).

Finally, we were able to demonstrate that adding another layer of electrospun

scaffold to constructs on top of the skin explants, does not inhibit cellular

migration from them. This is an important finding, as any skin replacement

technique, apart from the technical aspects of its application will need an

outline as to how it is to be dressed. Dressings form an integral part of wound

care and follows a specific sequence. In simplistic terms the first layer is usually

a non-adherent layer that does not disrupt ay developing wound islands on

removal for inspection, the second layer is an absorbent layer that allows

egress of exudate and the last aids to bind the first two, holding it in place,

 Our work has shown that by using another layer of electrospun scaffold in the

sandwich technique to ensure the skin explants are not displaced does not

result in inhibition of cellular migration. This can be one method via which this

technique is translated clinically. The first layer of electrospun scaffold will

form part of the new dermis and integrate with the underlying wound and be populated by cells from the skin explants. This can safely be protected by another layer of electrospun scaffold on top of the skin explants. This top layer of electrospun scaffold is advantageous as due to its porosity will allow wound exudate to be released, which prevents fluid accumulation and build up which can lead to failure of the technique. Furthermore, this top layer of scaffold can be designed using fast dissolving PGLA 50:50 which would degrade to allow inspection within 5-7 days to ensure that the technique has been successful and as a result, does not have to physically be removed which would facilitate the least amount of disruption to the underlying wound.

This chapter aimed to refine our technique of using skin explants to be combined with electrospun scaffolds as a one-step approach for wound healing. We applied some clinical principles to aid this, and overall have fulfilled the aim of retention of skin explants on scaffolds. However, these experiments have all illustrated that time is a key factor in the migration of cells when skin explants are placed on electrospun scaffolds. The *in vitro* model still does not reflect the exact surface that the scaffolds will be placed on *in vivo*.

Any wound bed, be it full thickness where all of the dermis is damaged or disrupted or partial thickness where some of the dermis is left behind will have a microenvironment consisting of inflammatory cells, cytokines, and growth factors that will facilitate the migration and proliferation of skin cells. These often come from the edges of the wound and the blood that is present on the wound bed, even if it is a full thickness defect. This explains why tissues that are well vascularized make for better wound beds for skin substitutes and grafts e.g. muscle compared to those that are not e.g. bone without periosteum. Despite the steps that we have taken in this chapter to optimize our construct, the skin explants are still not in the environment of a vascularized wound bed. As a result, migration and proliferation of cells takes too long a time than would be clinically acceptable for augmented wound healing.

The following chapter aims to address this by creating an *in vitro* wound bed in order to test how readily cells from the skin explants will migrate onto electrospun scaffolds in an environment that would be similar more to that of a debrided wound.

Chapter 7

Investigating the contribution of fibroblasts in the scaffold to epithelial cell outgrowth from skin explants

7.1 Introduction

Thus far we have outlined the process undertaken of developing a tissue engineered skin substitute consisting of a templated trilayer dermal scaffold to be cultured with skin explants as a cell source. Initial experiments demonstrated that the cells will migrate from the cut edges of the skin explant onto the fibres of the electrospun scaffold. Following this, various strategies were outlined to optimize the migration of skin cells out of the skin explants onto scaffold. These included using fibrin, altering the dermal thickness of the skin explants and using two layers of the electrospun scaffold with the explants in the middle.

These experiments were all conducted in vitro, and it was felt that it would be appropriate if the constructs better represented an in vivo wound bed model. The dermis has a central role to play in wound healing, and this is largely due to the extra cellular matrix (ECM) and the resident fibroblasts. The ECM can be thought of as a conglomeration of fibres forming molecules which provide a 3D framework of rigid proteins, non-fibre forming molecules which creates a charged osmotically active space and matricellular proteins that influence cell interactions(226). Collagen accounts for 77% of the fibre forming molecules in the skin, others include fibrin, fibronectin, vitronectin, elastin and fibrillin(227).

The main non fibre forming molecules are proteoglycans and GAGs, the most

common being hyaluronon, followed by decorin, versican, and

dermatopontin(228). The matricellular proteins do not contribute to ECM

structure as do the other two, but are critical to cell signalling. In the healing

wound, fibroblasts produce most of these components, and in turn they

influence the function of the keratinocyte(229). As a result, this autocrine-type

interaction between the fibroblast and the ECM can be viewed as a crucial

aspect of wound healing.

In all our experiments thus far, we have shown that the trilayer templated

electrospun scaffolds will support the migration and proliferation of epithelial

cells. We have established a technique of skin explant preparation, laying of

skin explants onto scaffolds and using fibrin as an adhesive to facilitate the

contact of the skin explants with the scaffold. Nevertheless, the skin explants

were still being cultured directly onto sterile electrospun scaffolds, with no

external or surrounding cell signalling proteins to stimulate migration and

proliferation of cells, the importance of which is outlined above. It was felt that

this did not mimic the in vivo micro-environment of a dermal wound, as the

eventual aim would be to use this technique in vivo, on a clean, prepared

wound bed which would contain the stimulus to propagate epithelial cell migration and proliferation.

In an attempt to do this, we therefore opted to culture the electrospun scaffolds for 48 hours with confluent isolated fibroblasts to allow for a prior fibroblast presence and release of the appropriate signalling factors on the electrospun scaffold. This aim of this was to create a micro environment which better represents the wound bed to further assess the performance of skin explants as a cell source on the electrospun scaffolds.

Additionally apart from the crucial role in wound healing, the use of precultured dermal scaffolds with isolated fibroblasts has been shown to be associated with the formation of more mature collagen bundles in the regenerating wound(230). The practice of preculturing fibroblasts on electrospun scaffolds has always been viewed as a method to aid optimisation of tissue engineered bioproducts for skin replacement. There are different approaches to this technique from culturing both sides of a scaffold with fibroblasts and keratinocytes(231) to culturing fibroblasts only on the under surface of co-polymer scaffolds and keratinocytes at the top (232). In this method, we propose the use of fibroblasts to simulate the wound bed conditions that our scaffolds would be placed on. Even if a wound is devoid of dermis, there will still be some background inflammatory process, which will

act as a stimulus to aid the integration of the scaffolds. Without these signals, it is very challenging to judge the true extent of cellular migration that would occur in vitro. These experiments aim to firstly produce a more accurate mimic of the wound bed and also use the knowledge from the previous chapters to optimise the cellular migration from skin explants on these electrospun scaffolds. As outlined previously, the use of isolated cultured cells is fraught with challenges in the clinical setting and we want to determine if a small skin explant, readily available in theatre would have a similar effect in a similar time frame. To do this, we isolated human dermal fibroblasts from donated skin as outlined in the methods. Once the cells were confluent, they were then seeded onto the PGLA (75:25) surface of the templated trilayer electrospun scaffolds. These were kept in culture for 48 hours to enable to the fibroblasts to adhere to the scaffolds and start proliferation. Once this was complete, the minced pieces of skin explants were added to the centre of the scaffold with fibrin (at a thrombin concentration of 2.5 I. U and fibrinogen at 18.75mg). These were cultured for another 48 hours before raising to an air liquid interface. Once raised, the culture media was changed every 48 hours and kept for a total of 14 days. Interim measurements were taken at day 7 for all samples. Quantitative analysis included Alamar blue and Sirus red for assessment of metabolic activity and collagen deposition. Qualitative analysis

included confocal immunohistochemistry to test for evidence of keratin from proliferating keratinocytes combined with DAPI to illustrate the the location of the cells in relation to the keratin deposited. Furthermore, we used fluorescence microscopy to obtain images of the constructs after incubating them with Ki67 which is a marker of proliferation to assess the location of these cells on the electrospun scaffold. The aim of this was to determine the location of these cells that were actively proliferating and determine if there was tendency to occupy the niches of the electrospun scaffold that were templated on them for this purpose. Furthermore, we stained the constructs for collagen 4, as another assessment to visualize the location of the collagen deposited by the proliferating fibroblasts.

7.2 Use of AlamarBlue assay to demonstrate cell outgrowth from explants

Absorbances for skin explants combined with fibrin and those without fibrin seeded on fibroblast impregnated templated electrospun scaffolds were read and the absorbance for plain scaffolds were subtracted from these values for each time checkpoint of day 0,7 and 14 (Figures 7.1 and 7.2). Additionally, we compared the metabolic activity of these constructs to templated scaffolds cultured with explants over a 14-day period with no fibrin or pre culture with fibroblasts. Furthermore, as a positive control we used tissue engineered skin

(isolated fibroblasts and keratinocytes cultured on DED) to determine the impact, if any of pre culturing fibroblasts and using fibrin made to the outward migration of skin cells from the cut edge of the skin explants.

It was demonstrated that skin explants combined with fibrin cultured on fibroblast impregnated scaffolds were viable throughout the 14-day testing period. Cellular metabolic activity in constructs precultured with fibroblasts and fibrin demonstrated significantly increased metabolic activity compared to using co-culture scaffolds without fibrin and when the explants were simply laid onto the scaffolds without preculturing them with fibroblasts. Furthermore, it was also noted that co-culture of scaffolds with fibrin and explants gave a significantly increased amount of cellular metabolic activity compared to using DED scaffolds seeded with isolated cells. As demonstrated in earlier chapters, constructs where the explants were simply seeded on the plain scaffolds resulted in the least amount of metabolic activity as demonstrated in Figure 7.1.
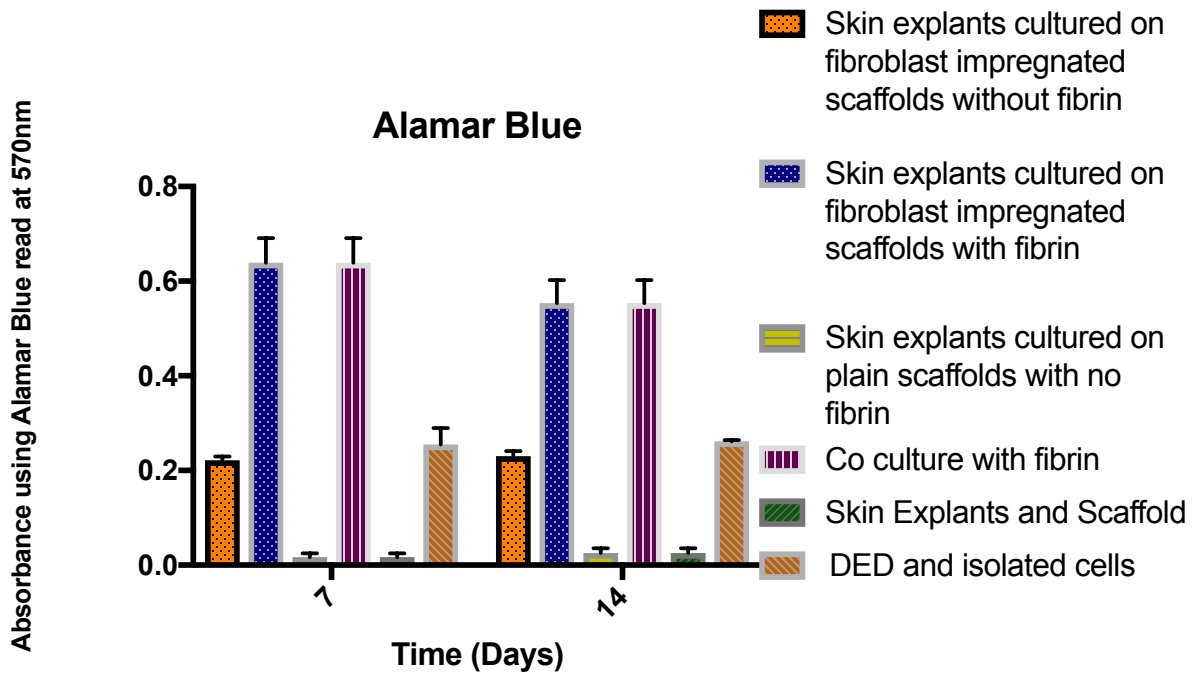
Figure 7.1 *Metabolic activities of skin explants combined without fibrin (orange bars) and fibrin (Purple bars) on fibroblast impregnated scaffolds compared to skin explants cultured on plain templated electrospun without fibrin over a14-day experimental period as determined by absorbance of AlamarBlue stain (Mean±SEM).*

7.3 Use of Sirius red assay to assess collagen production

At day 7, 14 Sirius red assays were undertaken to estimate the total collagen produced overtime by the co-culture/fibrin constructs compared to skin explants seeded on plain scaffolds. The aim was to quantify the ability of the electrospun scaffolds to support cellular function using collagen production by fibroblasts as a marker. The absorbance of Sirus red is plotted in Figure 7.3

minus the absorbance of plain scaffolds. Although at all time checkpoints, the

amount of collagen produced was more, this was not statistically significant

(P=0.2) because of the considerable variation between the replicates.
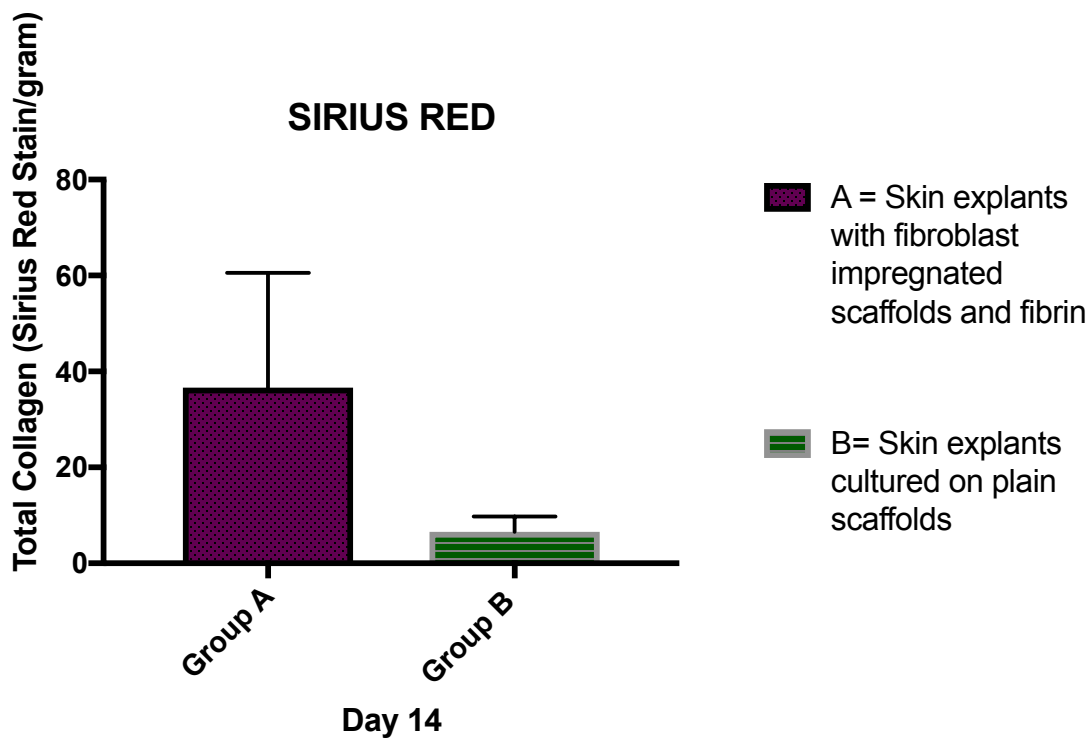
Figure 7.3: Sirius red staining after 14 days comparing a: Skin explants cultured with fibroblast impregnated electrospun scaffolds and fibrin (purple) and b Skin explants cultured on plain scaffolds. (N=3±SEM).

7.4 Identification of keratinocyte outgrowth using immunohistochemistry for pancytokeratin and confocal microscopy.

At the end of the 14-day culture period, constructs containing skin explants

seeded with fibrin on fibroblast impregnated scaffolds were stained for

pancytokeratin as outlined in the methods above. In previous experiments, H&E stains were used to visualize the migration of the epithelial cells along the scaffold, however given that the wound bed model demonstrates a significant amount of cellular migration from the cut edges of the electrospun scaffold, it was thought that further cellular characterisation using immunohistochemistry would be of benefit. The aim of this assessment was firstly to determine if the keratinocytes were proliferating on the electrospun scaffold and producing keratin, which is the main protein of the epidermis. The only source of keratinocytes in these constructs were from the skin explant seeded on the scaffolds. We hypothesized that by pre-culturing fibroblasts onto the scaffolds, this would aid the outward migration of keratinocytes from the skin explants to populate the scaffolds. Simultaneously we stained the same constructs using DAPI to visualize the cellular nuclei around the areas stained with pancytokeratin.

Confocal imaging was chosen as the templated scaffolds were not flat 2D structures, but had an undulating pattern in the middle layer. This technique allowed for varying depths of the middle layer of the electrospun scaffold to be imaged.

7.4.1 Assessment of cellular migration by staining nuclei for DAPI and imaging using confocal microscopy

Figure 7.4 demonstrates the various depths that the cells extend to in the template as illustrated by the blue stained nuclei. Figure 7.4 B demonstrates the depth in detail, which illustrates that the cells penetrated the entirely of the scaffold. Furthermore, we were able to visualise the outward migration of cells from the cut edge of the explants (Figure 7.5) which provides further evidence of the outward migration of cells from the explant.
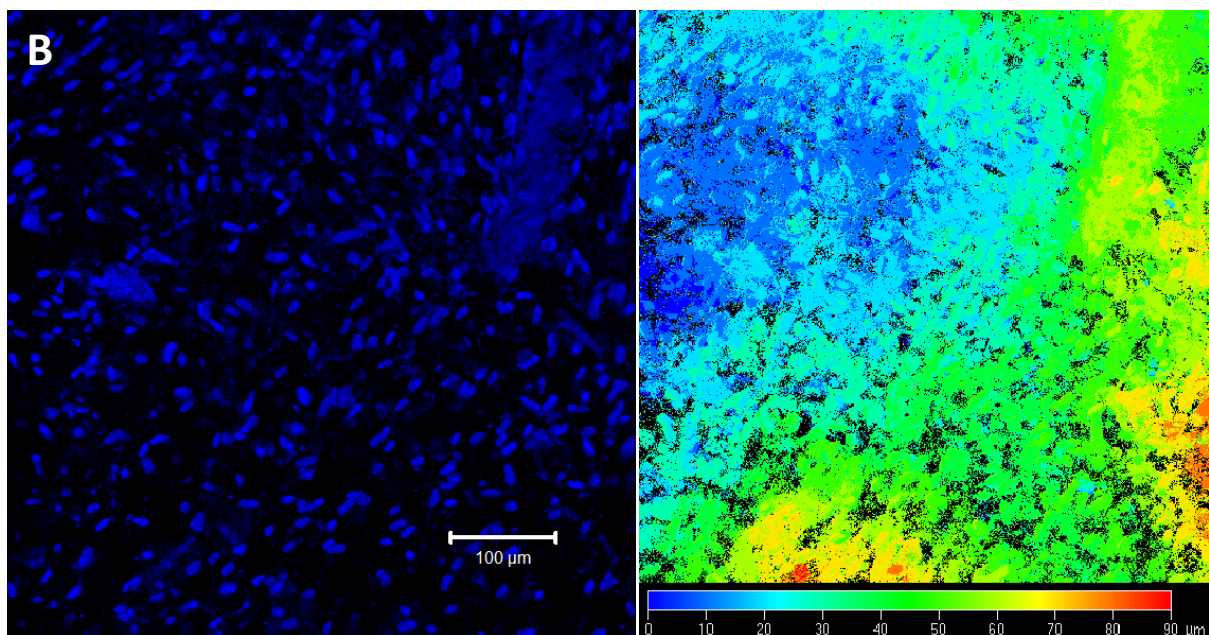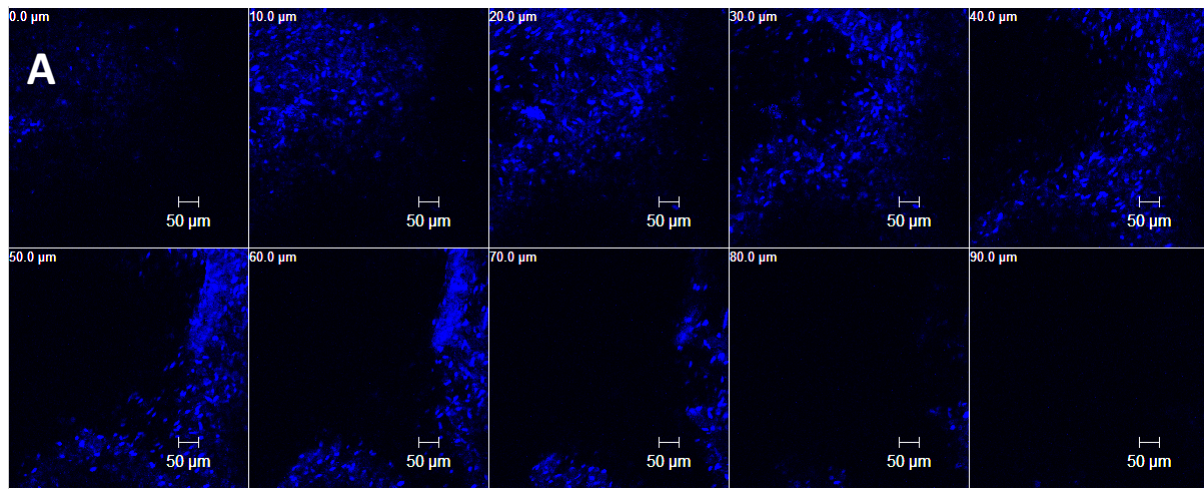
Figure 7.4 A: *Confocal imaging of 14 day old constructs of skin explants with fibrin cultured on fibroblast impregnated templated scaffolds using DAPI which illustrates from left to right the presence of nucleated cells throughout the thickness of the scaffold, left being the most superficial aspect and the far most right being the deepest aspect. B: Magnified view of Figure A illustrating the vertical penetrance of the epithelial cells through the templated scaffolds.*

A

0.0 μm · 30.0 μm · 60.0 μm · 90.0 μm · 120.0 μm · 150.0 μm
180.0 μm · 210.0 μm · 240.0 μm · 270.0 μm · 300.0 μm · 330.0 μm
360.0 μm · 390.0 μm · 420.0 μm · 450.0 μm

Skin explant on corner of scaffold

B

Skin cells seen migrating from the cut edge of the skin explant onto the scaffold

200 μm
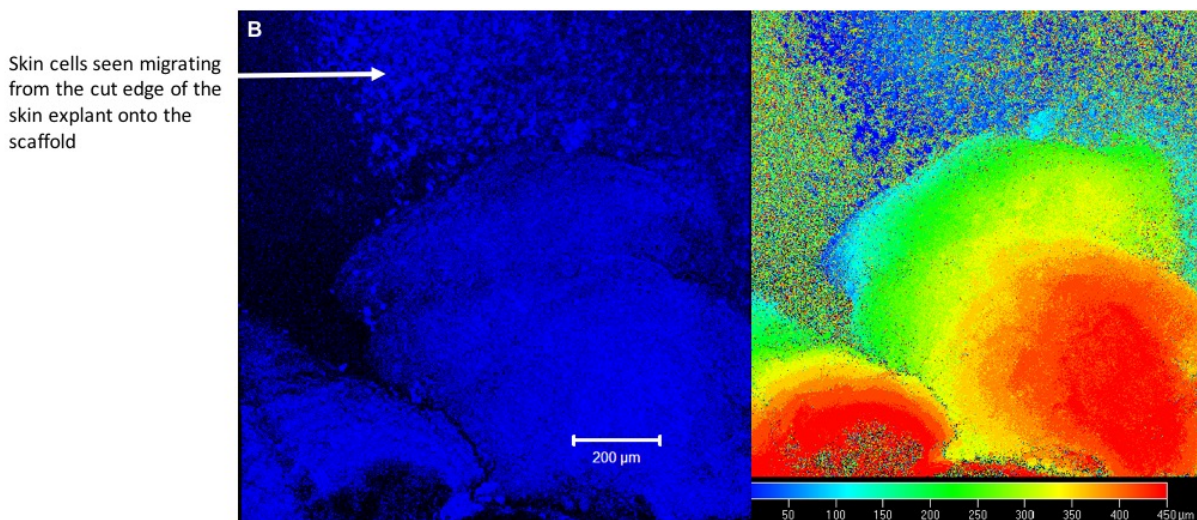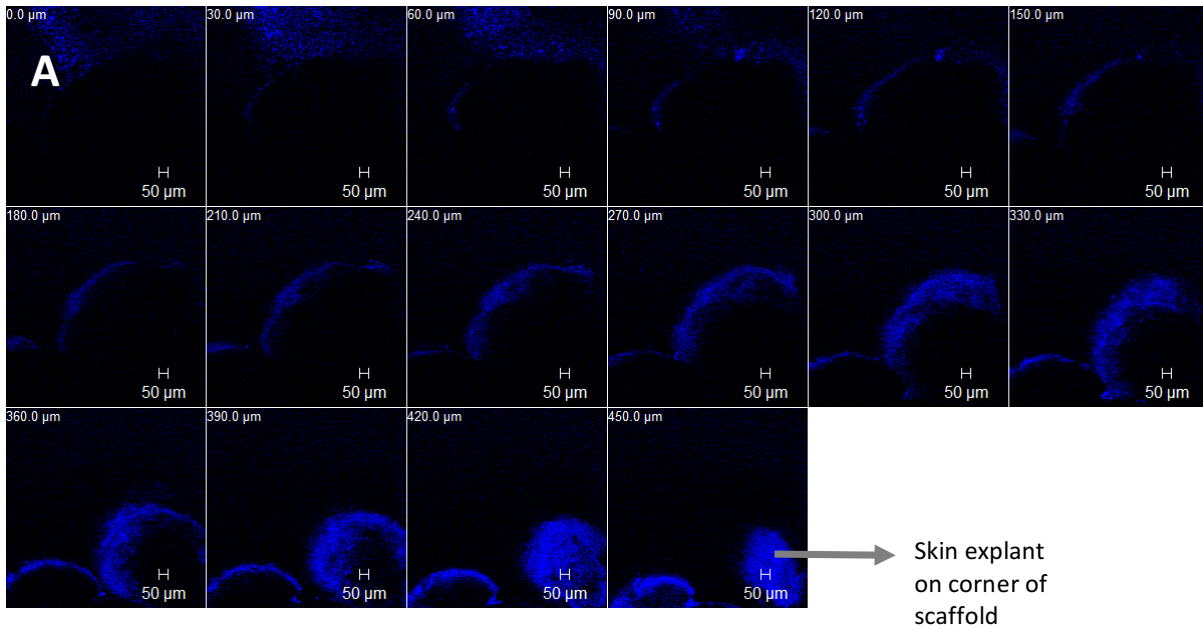
Figure 7.5 (A) Confocal imaging of 14 day old constructs of skin explants with fibrin cultured on fibroblast impregnated templated scaffolds using DAPI which illustrates from left to right the presence of nucleated cells throughout the thickness of the scaffold, left being the most superficial aspect and the far most right being the deepest aspect. These images also demonstrate the skin explant (labelled) and the cells that are migrating from the edge *(B)*.

7.4.2 Pancytokeratin Staining combined with Confocal Imaging

The above constructs were stained using pancytokeratin and imaged to confirm the deposition of keratin in areas just adjacent to the implant, which is an indicator of keratinocyte migration and proliferation. This was an important stain as it proves that keratinocytes, the cells that make up the epidermis will proliferate on the templated trilayer electrospun scaffold. Figure 7.6 illustrates that the area just adjacent to the skin explants contained cells that have migrated onto the electrospun scaffold that overlap the areas stained for the pancytokeratin (green). It also demonstrates that keratinocytes proliferated the depth of the scaffold which increased its surface area. Figure 7.6 C illustrates this more closely, which further confirms that the cells are producing pancytokeratin, just beyond the explant.
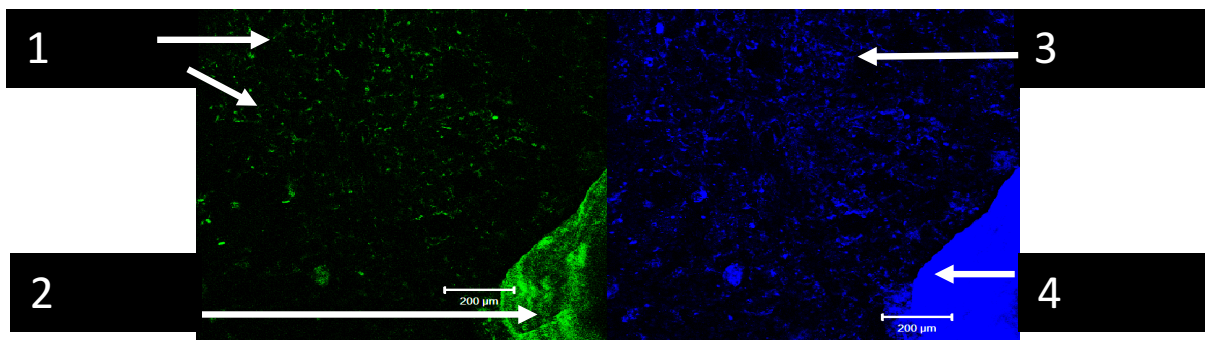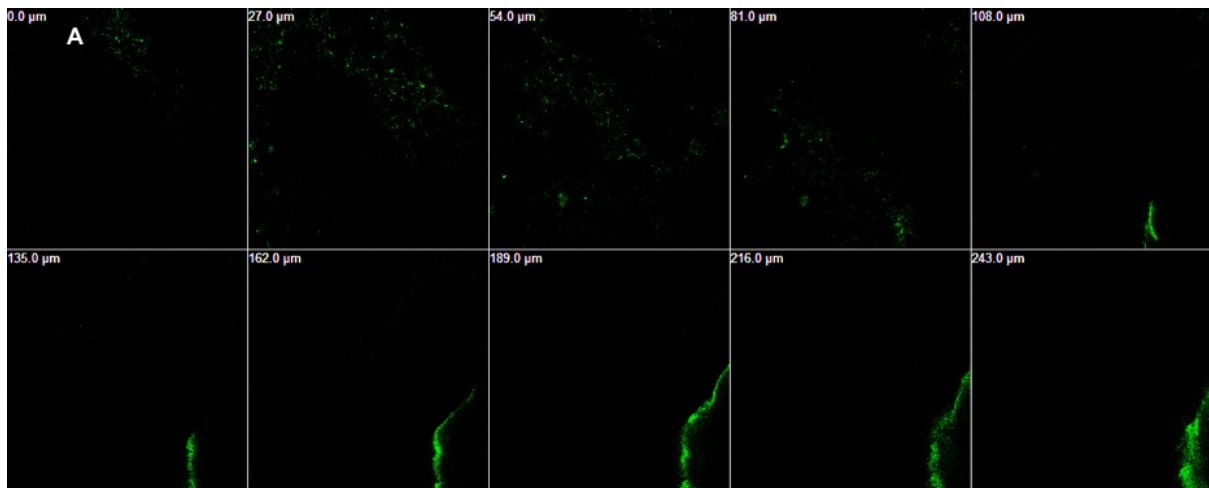
Legend

1. Green areas of pancytokeratin staining adjacent to the explant
2. Skin explant stained green with pancytokeratin
3. Blue areas of DAPI staining of cellular nuclei adjacent to the explant
4. Skin explant stained Blue with DAPI

Figure 7.6 *14 day cultured samples with skin explants combined with fibrin on scaffolds cultured with fibroblast impregnated trilayer electrospun scaffolds stained for pancytokeratin and imaged using the confocal microscope. A shows the stain distribution throughout the depths of the trilayer scaffold, B shows area of cellular staining and migration just adjacent to the skin explant. Scale bar = 200µm.*

7.5 Scanning Electron Microscopy

These images were taken for fixed samples at day 14 of the pre-cultured scaffolds with fibroblasts /fibrin /skin explant constructs. The images clearly show the skin explant on the templated scaffold (Figure 7.7A). On further magnification (Figure 7.7B) the fibrin strands randomly orientated on the surface of the scaffold is seen, together with adjacent areas of deposition of products of cellular migration and proliferation from cells just adjacent to the skin explant on the scaffold. This provides further evidence that the cells are not only able to leave the explants and migrate along the fibres of the scaffold but also proliferate and manufacture essential proteins like keratin and collagen, which are the key materials that make up the epidermis and dermis respectively.
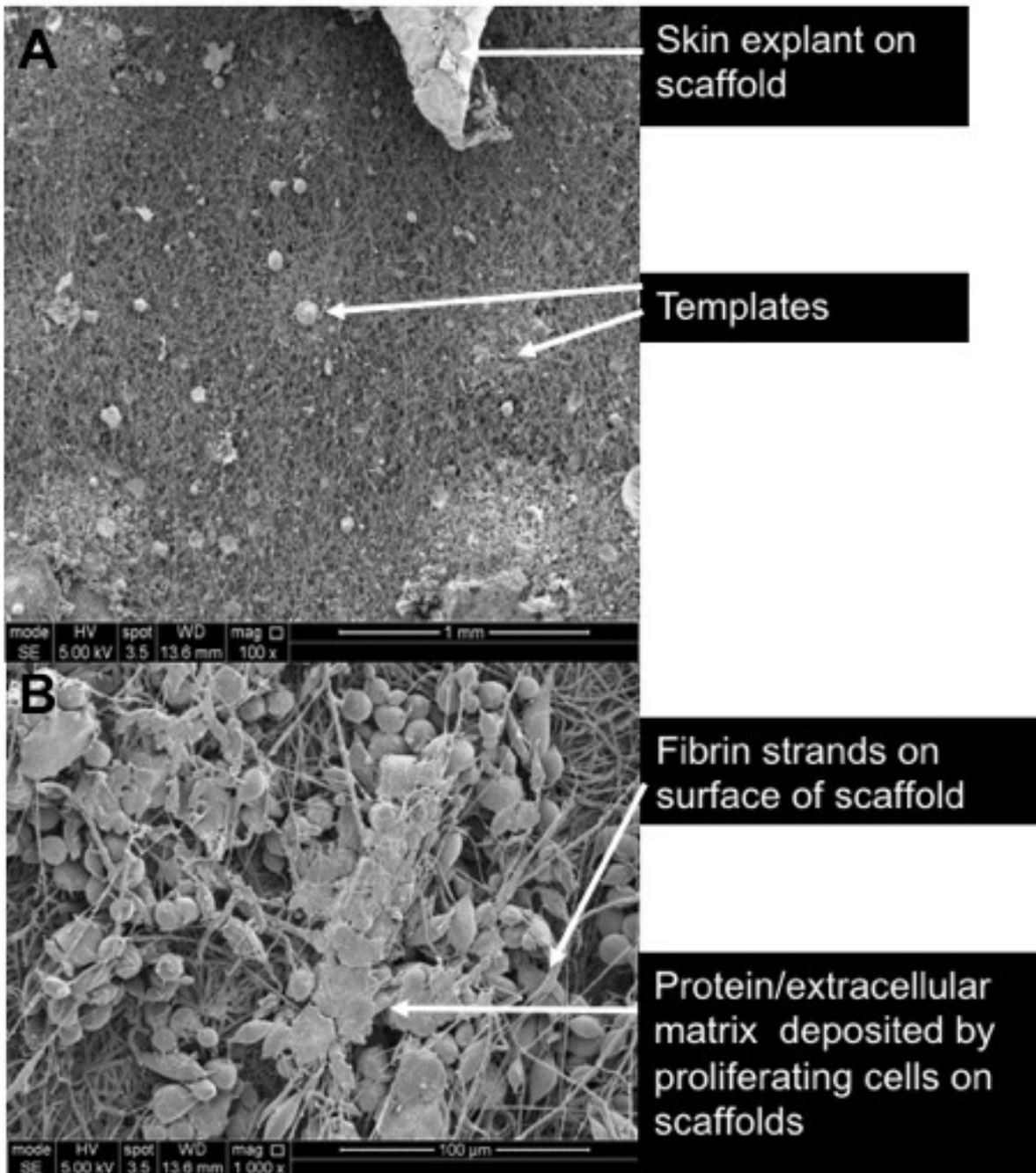
Figure 7.7 A *SEM image of skin explant with fibrin on precultured scaffolds with fibroblasts illustrating the edge of the explant on the templated scaffolds. Figure 7.7 B shows higher magnification of an area just adjacent to the skin explant containing fibrin stands and extracellular matrix material deposited by the proliferating fibroblasts.*

7.6 Immunohistochemistry for Collagen IV and Ki67 using Epifluroscent microscopy.

The aim of these experiments was to observe the deposition of collagen on the electrospun scaffold as another method to prove that fibroblasts were able to migrate and proliferate along the fibres of the scaffold. For these experiments we seeded isolated cells on either side of the trilayer templated PGLA (75:25) and PHBV scaffolds and kept the constructs in culture at an air liquid interface for 14 days. In addition to this we also assessed the scaffolds that were cultured with isolated fibroblasts for 48 hours prior to application of skin explants cultured for the same two-week period at an ALI. At the end of the culture period samples were fixed in 3.7% formaldehyde and cryopreserved. 7 μm slices were glass mounted and stained with collagen IV (details outlined in methods). After 24 hours' samples were stained with DAPI to visualize adjacent cell nuclei and then rehydrated. For collagen IV, 2D images were obtained using an Olympus IX73 Epifluroscent Microscope using the following excitation and emission wavelengths:

- DAPI: λex 385 nm/λem 461 nm.

- TRITC: λex 545/25 nm/λem 605/70 nm.

- FITC: λex 488 nm/λem 519 nm.

Our images revealed firstly that collagen was present on the fibres of the electrospun scaffold when stained with collagen IV. DAPI staining showed the concurrent nuclei of fibroblasts along the collagen stained electrospun scaffold fibres. The trilayer constructs were not visible in these images as the process and solvents of cryofixation and sectioning, damaged some of the inherent trilayer structure. Nevertheless, we were able to visualize areas of collagen deposition along the scaffold, where the fibroblasts were seeded (Figure 7.8)

Top and middle
layers of the scaffold
stained with collagen
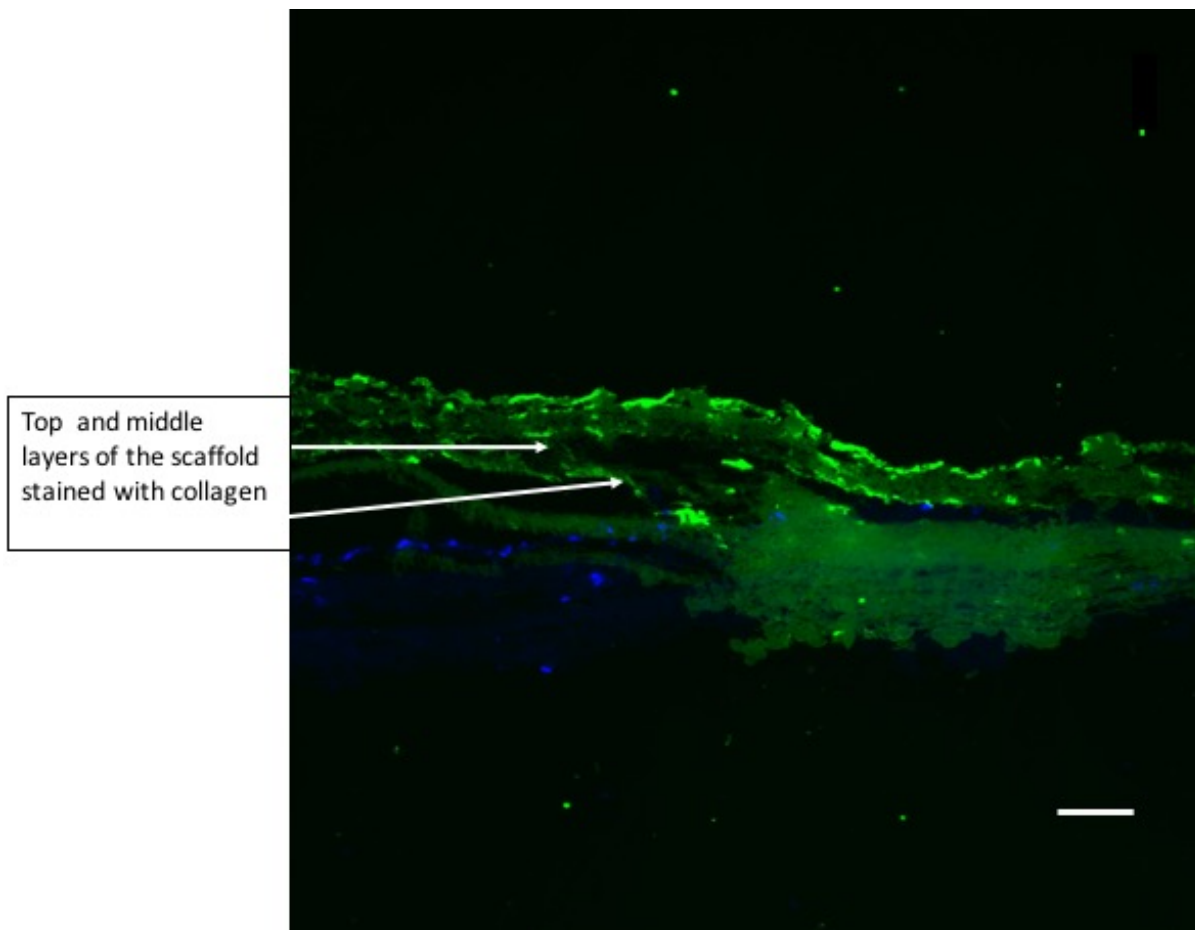
Figure 7.8 *Collagen immunohistochemistry of templated electrospun scaffold*

illustrating collagen deposition along the top and middle layers of the

electrospun scaffolds. Scale bar x 0.2mm (x100).

Furthermore, we wanted to assess the effect if any, of changing the

topography of the scaffold, whether going from being straight to undulating

had on the organization and distribution of cells. Specifically, we wanted to

assess its effect on the proliferating cells, as the design intended to stimulate

the rete ridges, which is one of the areas that cells with proliferative capacity

in the skin tend to occupy in vitro. To do this, we cultured isolated fibroblasts

and keratinocytes on either side of the templated trilayer scaffold as above

and performed immunohistochemical staining using Ki67 of formalin fixed 7µm

sections followed by imaging using the Epifluroscent microscope as described

earlier. Ki67 is a nuclear protein that is expressed in the cell throughout the

various stages of the cell cycle but is maximally expressed during the G2 or

mitotic phase(233). It is often used as a marker of cellular proliferation as it is

not detected in G0 cells, but increases from G1 through mitosis(233). As a

result, the Ki-67 antibodies were deemed to be useful in identifying and

imaging the location of the maximally proliferating cells in our constructs. DAPI

was also used to visualize cellular generally to obtain a composite picture of

cellular location. The figures indicate that proliferating cells were seen in our

samples and tended to be found in the troughs of the template. Other cells

were seen scattered throughout the scaffold (Figure 7.9).

Proliferating cells conglomerating in the trough of the templated scaffolds (middle layer)

Third layer of scaffold-representing the neodermis of PGLA 75:25 .
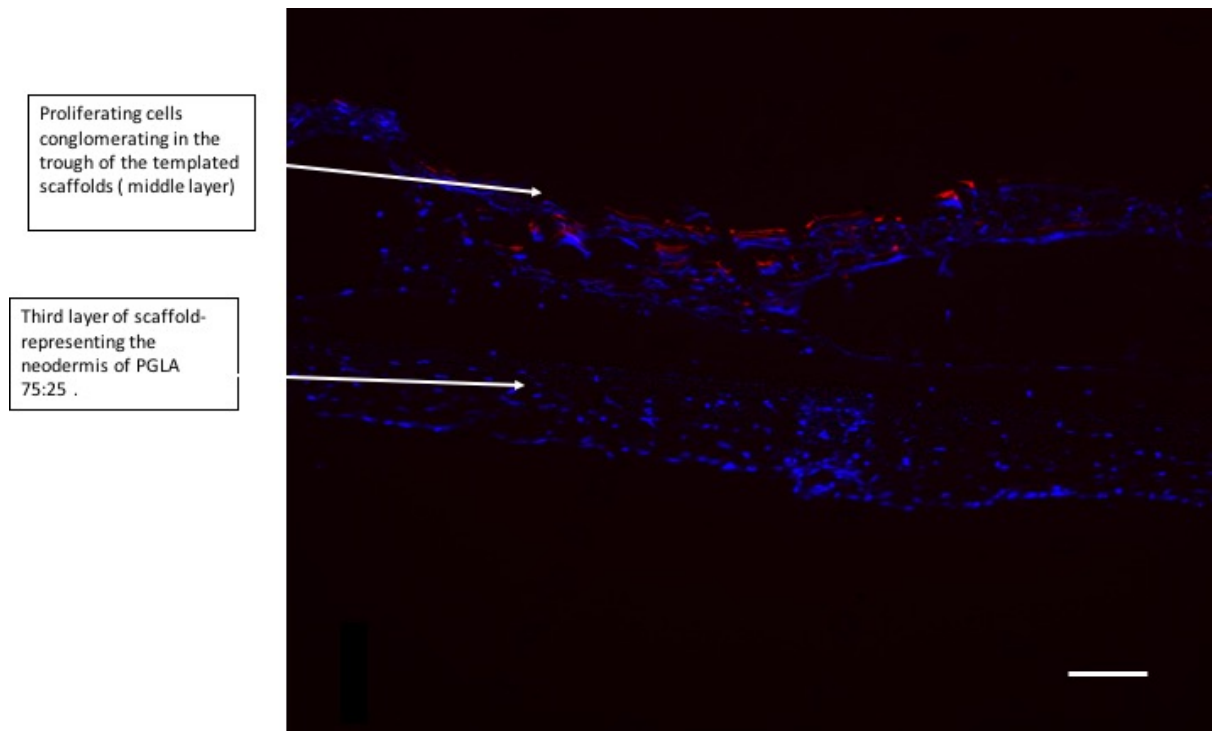
Figure 7.9 *Ki67 immunohistochemistry staining showing proliferating cells clustering along the trough of the niche, illustrating the potential benefit of templating the electrospun scaffolds. Scale bar 0.2mm (x100).*

7.7 Discussion

This chapter aimed to combine the steps outlined in previous chapters to optimise the migration of cells from skin explants onto the electrospun scaffolds in the context of a wound bed model. To create this wound bed in the in vivo setting, we seeded fibroblasts, the main cell of the dermis, with the aim of releasing signals to chemo attract cells out the skin explants and onto the electrospun scaffolds for 48 hours prior to application of skin explants and fibrin glue. These were then cultured for a further 14 days at ALI. These experiments were essentially the final checkpoint, to test the validity of the

technique before it can be progress to being tested in animal models.

Quantitative analysis using Alamar blue and Sirius red, revealed that these wound bed models had a significantly greater amount of metabolic activity and collagen deposition versus culturing the skin explants onto plain electrospun scaffolds. This effect is to be expected as per unit of scaffold, there are more cells and moreover the ones that result in collagen formation and can be argued that it is not coming from the skin cells that have migrated out the skin explants. However, this model is the closest in vitro prototype that will be translated into in vivo studies and represents the situation that will occur once these electrospun scaffolds are placed onto an active wound bed.

Imaging the constructs using confocal imaging and immunohistochemistry using DAPI, pancytokeratin allowed us to observe the anatomy of the scaffolds seeded with fibroblasts and skin explants to a much greater detail than H&E staining confirmed two important technical points that H&E were not able to provide as the process of H&E can be quite destructive to scaffolds and do not always illustrate clearly the relationship between the fibres and cellular migration. Firstly, it shows that the cells populate the scaffold throughout its depth and not just along the surface onto which it is placed. Additionally, it proves that there are cells that migrate from the skin explants and some of these were keratinocytes. This is an important fact as the skin explants in these

models were the only source of keratinocytes considering the scaffolds were pre seeded with fibroblasts. This provides further evidence that skin explants can be used as a cell source to populate the scaffolds.

The SEM images, provide similar information however it allowed us to observe the strands of collagen and extracellular matrix produced by proliferating fibroblasts. It is unknown whether this is from fibroblasts that were used to pre-seed the scaffolds or from those that migrated from the skin explants either way, the edges of a full thickness wound bed will have a population of fibroblasts that will migrate into the wound to have a similar effect as our model.

Collagen immunohistochemistry using the Epifluroscent microscope revealed that collagen was not only deposited on the top layer but also on top of the middle layer of the electrospun scaffold. This is an important finding as although it is expected that fibroblasts will populate the scaffold to form the neodermis, the middle layer is intended to mimic the basement membrane. Skin conforms to a 3d macrostructure, with defined layers that work synergistically to allow the skin to function as an organ. Yet most tissue engineered substitutes are 2D, flat sheets of scaffold. The basement membrane is an important part of the skin; it aids secure attachment of the overlying epidermis containing keratinocytes. Another important feature of a

basement membrane is that it resists cellular movement across it, to allow for

organisation into an epidermis and a dermis. The basement membrane is

made from specialized extracellular matrix which facilitates these roles(4).

Furthermore morphological studies have revealed that the basement

membrane is predominantly collagen IV in humans(234). In our constructs the

central layer that aims to mimic the basement membrane was made from

nano-porous /nano fibrous PHBV interwoven with micro-porous poly L-lactic

acid microfibers on either side to avoid separation of the polymers once

cultured in media. Previous H&E images (chapter 4, Figure 5.4B) revealed that

this layer is impermeable to cells, which is an essential feature of the basement

membrane. Therefore, to summarize thus far, we have created an

impermeable basement membrane, which will allow for a distinct epidermis

and dermis.

In addition to having this definable structure, we wanted to incorporate its 3D

topography in the form of rete ridges which are the peaks and troughs that are

present within it (Figure 1.2 Introduction).

This topography not only aids epidermal and dermal attachment on either side

but also creates to a unique microenvironment to house proliferative cells.

These cells predominately tend to occupy the troughs of the rete ridges(13).

The ability to maintain the proliferative capacity of skin must be considered

when designing any material that aims to be its replacement, which is the intended aim of this model. Ki67 immunohistochemistry revealed that there were indeed proliferative cells that occupied the troughs of our template scaffolds. The limiting feature here is that it is not clear which cell source this is i.e. epidermal or dermal. However, we have established a technique by electrospinning which not only allows a trilayer scaffold to maintain its structure throughout our experiments, but also offers a niche for proliferative cells that migrate from the skin explants onto the scaffolds. This is in the long term we believe is advantageous for the long term aim of this technique, as a facilitator for skin regeneration, whereby the electrospun scaffold integrates with the underlying wound bed and it is populated by cells migrating from the skin explants. These proliferating cells will produce the ultrastructure of a new dermis by laying collagen and extracellular matrix and an epidermis by way of keratinocyte proliferation. Over time, the polymer will hydrolyse leaving behind the patient's new skin. This is the fundamental principle of tissue engineered dermal substitutes which contrasts current clinical products that are in use. The dermal scaffolds are all made of polymers eg bovine collagen in Integra, with no intention to degrade and facilitate regeneration of the patient's own skin. Although this is acceptable and has been in use for many years, the technology has been refined from advances in biomaterial sciences

to manipulate synthetic polymers to facilitate process, which in the future will

be generation next of skin replacement.

Chapter 8

Conclusions and Future Directions

8.1 Current clinical problem

The management of large areas of full thickness skin loss is still a reconstructive challenge. Skin replacement has evolved with research, improved technology and most importantly a better understanding of wound healing. There are many variations on the use of autograft harvest and application and the use of cadaveric allograft has been commonly used over the years. However, as assessment of clinical outcomes evolve and the definition of a good outcome following skin reconstruction changes from simple survival to functional, a questioning approach to traditional techniques has led to the development of biomaterials and tissue engineered biomaterials for skin replacement. Globally, burn injuries are the 4th most common cause of trauma accounting for over 300,000 deaths each year(235). Unfortunately, low and middle income counties bear 95% of this burden and access to burn treatment and follow up care is limited by the high cost of care and adequate health care infrastructure(236).

Discussing the use of allograft cadaveric skin is interesting in that it provides an immediate barrier to microorganisms and prevents evaporative losses. It can be used as an immediate temporary dressing or it can be used such that the cadaveric donor dermis remains in place providing a decent wound bed over which to graft split thickness autograft or laboratory expanded keratinocytes

can be applied(78). In practice what has limited its use in the UK at the time of writing is its availability. Any human tissue gives the risk of viral disease transmission, tissue banking practices are established to mitigate this risk by doing extensive screening of blood samples from donors, including lifestyle questionnaires and storage of tissue prior to its release.

In Holland, the Dutch Burn Centres have collaborated to establish a skin bank which provides screened donor allograft known as Euroskin to burn centres throughout Holland and even to the UK when requested. The NHS blood and tissues organisation which is tasked with providing cadaveric skin to burn centres in the UK has not achieved a similarly reliable supply of cadaveric skin for UK burns centres.

The use of donor skin in theory provides a simple to use, cost-effective dressing to manage major burn patients. Unfortunately, in many countries where it could benefit burns patients there are cultural and religious objections to the use of human skin. Furthermore, the infrastructure of tissue banks to provide low risk donor skin has simply not been organised. This is an example of a product which works clinically (and has been the basis of many published studies demonstrating its clinical usefulness) but availability dictates whether it is actually used by surgeons. Alternative approaches to using

cadaveric skin have led to the development of sophisticated biological biomaterials such as Integra and Matriderm.

A long term solution could potentially lie in the field of synthetic biomaterials; free of animal and human products, that are long lasting, off the shelf and readily available for use in these settings. Most importantly, they need to be affordable. Currently used skin substitutes in the United Kingdom eg Integra and Matriderm do not fulfil these criteria. Integra has been used in the treatment of acute and chronic burn wounds since the 1980s. It is composed of bovine collagen to which a silicon sheet is bonded to provide a temporary barrier layer (3). Many studies have shown excellent clinical results using Integra. Its routine use is challenged by the cost per sheet, and the risk of infection. Many surgeons have noted that unless there is meticulous wound bed preparation, the anaerobic environment provided by the silicon barrier seems to facilitate rapid development of pseudomonas bacteria. In practice considerable care must be taken to prepare and clean the wound bed prior to its application. Despite being commercially available as an off-the-shelf product it is not economically viable in the current NHS and in many developing countries, where large burns are still prevalent. This highlights the need to further evolve our concept of biomaterials, as the answer to a lot of these issues may lie with designing materials which are inherently not

expensive and can be made readily available and which are relatively straightforward to use in theatre.

Of necessity this means that any project that requires cultured live cells is inherently more difficult to plan, organise and fund irrespective of whether cell-based products are clinically effective. The production of tissue engineered skin is arguably the oldest and best established of all tissue engineered products. Early examples of laboratory expanded keratinocytes being used to provide barrier cover for badly burned patients dates back to the early 1980s.

A search of the published literature will reveal a wealth of studies in which laboratory expanded cells have been used to give benefit to patients. For example, CEA provided the first tissue engineered approach to skin replacement. A great deal has been learnt in developing and evaluating these techniques. However, at the time of writing it is clear that issues of availability and ease of use and cost ultimately determine which published clinically effective methodologies survive the test of time.

In this study we sought to combine thin split thickness skin grafts, cut into many small pieces with a synthetic dermal substitute which could be used in a one-step approach in theatre to regenerate split thickness skin in an approach that did not require cell isolation or expansion using the patient's own cells.

Skin explants have been used clinically in the form of Meek Grafting and as part of traditional fibroblast culture using the principle that epithelial cells will migrate from the cut edges of the tissue.

A similar approach was developed from the MacNeil laboratory in the treatment of scarred corneas which have occurred as the result of limbal stem cell deficiency. The approach which has been used for the past 17 years has been to take a small biopsy of tissue from the unaffected limbus in the contralateral eye, expand cells under cleanroom conditions and graft it onto the scarred eye. A major simplification of this technique now only requires a biopsy of limbal tissue harvested from the contralateral healthy eye, minced into several tiny pieces and then spread onto amniotic membrane (scaffold). Using fibrin glue to hold the pieces in place, this was grafted onto surgically prepared corneas in a single theatre session (7). Within a few weeks epithelial cells are noted to have grown out from the explants to form a stable cornea in situ. This simplification has meant that many more ophthalmic surgeons can offer it to patients. This study highlights that for small areas of tissue reconstruction it is possible to combine tissue biopsies to regenerate new epithelium in situ. This is what we are aiming to achieve for skin epithelium regeneration.

Against this background this research seeks to combine very thin split thickness skin harvested in theatre with an off-the-shelf electrospun biodegradable membrane which can help promote rapid regeneration of split thickness skin. The design for this membrane is that it would have inherent 3D features within them to mimic aspects of the basement membrane and rete ridges. Scaffolds were also designed to degrade within 2-3 months leaving new, viable tissue in place.

As part of this also we want to assess the role of fibrin as a biological stimulator of the migration of skin cells from the explants onto the scaffolds as was found to occur with the migration of corneal epithelial cells from limbal tissue explants for corneal regeneration (8). Fibrin is an important blood component responsible for haemostasis and is often used surgically as tissue glue. It can be prepared from autologous plasma and is also available as an approved product for surgical use.

Thus we aimed to have a similar model for skin. Clinically this would translate as follows: a small biopsy of the patient's skin would be taken and minced into very fine explants. This would then be combined with the electrospun scaffold and placed onto the surgically prepared wound bed in combination with fibrin. It is expected that fibroblasts and keratinocytes will migrate along the fibres of the scaffold without the need for culturing cells in the laboratory and further

procedures in theatre. The commercial success of tissue-engineered products depends on their ease of use and their availability as well as their costs for widespread uptake. The approach we have described could become a key addition to the plastic surgeon's armamentarium in treating full-thickness skin defects to improve manage patients with extensive burns to achieve rapid skin cover.

The work to achieve this was described using 4 experimental hypotheses which translated into four experimental objectives as listed beneath

The hypotheses to be tested were:

1. Culturing skin pieces on a trilayer scaffold consisting of micro/nano fibres will lead to better epithelial-stromal organization than would be achieved on a scaffold consisting of microfibers alone

2. Inclusion of features that approximate the dimensions of the rete ridges will help in the outgrowth of epithelial cells into the electrospun scaffolds.

3. The inclusion of fibrin as tissue glue will act as a stimulant for epithelial outgrowth into these synthetic scaffolds.

4. The presence of fibroblasts precultured in synthetic scaffolds help stimulate the outgrowth of epithelial cells from skin explants

Following on from these the experimental objectives were:

1. Evaluation of how a novel method for spinning a basement membrane substitute can encourage epithelial-stromal organisation.

2. Investigation of whether the inclusion of features that approximate the dimensions of rete ridges will help in the outgrowth of epithelial cells into epithelial cells into the electrospun scaffolds.

3. Assessment of whether the inclusion of fibrin as tissue glue will act as a stimulant for epithelial outgrowth into these synthetic scaffolds.

4. Evaluation of whether inclusion of fibroblasts in the templated trilayer PGLA 75:25 / PHBV electrospun scaffolds will promote epithelial cell outgrowth in the presence and absence of fibrin.

This chapter now summarises to what extent the objectives and were achieved and the hypothesis supported or not.

8.2 Objective 1: The use of trilayer micro/nano electrospun scaffolds to better encourage epithelial-stromal organisation vs microfibers alone.

We aimed to create a novel method of spinning a basement membrane substitute to see if this would promote epithelial stromal organization into definable layers of the skin compared to when using microfibers alone. To do this we spun trilayer electrospun scaffolds, made using a combination of PGLA

75:25 (microfibrous and microporous) and PHBV (nanofibrous and nanoporous). The trilayer were shown to be easy to handle with similar mechanical properties to human skin. We chose these polymers based on previous work from the MacNeil laboratory whereby PGLA 75:25 was shown to degrade in a time period that matched the properties of a healing wound(124). PHBV was chosen for its nanoporous/ nanofibrous properties and ease of integration with PGLA 75:25, such that it made a good basement membrane substitute without delaminating from the PGLA 75:25 during experiments. Monolayer scaffolds of PGLA 75:25 supported cellular migration, however the fragility of the scaffolds made for difficult handling and the mechanical properties were not as close to skin as much as the trilayer scaffolds. We were able to achieve definable layers in the trilayer scaffold which resulted in a top layer of keratinocytes, a middle layer devoid of cells and an underside layer consisting of fibroblasts. In conclusion this experimental objective was partially met as although the handling properties of trilayer scaffolds were superior to the monolayers there was more cellular activity and proliferation in the monolayer constructs.

8.3 Objective 2: The inclusion of features approximating to rete ridges to create stem cell niches will aid in the outgrowth of epithelial cells onto scaffolds

The aim of this was to template the PHBV layer with a honeycomb like pattern to even further mimic the natural topography of the epidermal-dermal interface which is undulating in nature known as the rete ridges. These areas form microenvironments or stem cell niches which act to house the cells that are key in skin regeneration. We were able to use metal templates to successfully create these undulating patterns that were maintained throughout the experiments however in comparison with non-templated trilayer scaffolds were no significant differences in cellular activity and collagen production when skin cells and explants were cultured onto the scaffolds for comparison. Although there was some evidence that these ridges housed cells of a higher proliferative capacity on Ki67 staining the exact origin of these cells was not clear.

Thus in summary we are uncertain regarding the value of the templates in our model in terms of benefit but they certainly did not hinder the migration of isolated cells or those from skin explants throughout the scaffold.

8.4 Objective 3: The use of fibrin glue as an adjunct to stimulate the outgrowth of skin cells from skin explants onto scaffolds.

Our initial experiments revealed that the trilayer electrospun scaffolds were able to support the migration and proliferation of isolated keratinocytes and fibroblasts. However, when skin explants were used as a cell source, they did not proliferate to the same extent in the given time period as those of isolated cells. Given that one of the aims of this research was to use skin explants as a cell source we wanted to achieve a similar rate migration to that of seeding isolated cells onto scaffolds. To do this we postulated that continuous contact of the skin explants with the fibres of the scaffold is essential, which was difficult to achieve using in vitro conditions with cell culture media continuously displacing the explants.

As a result, we hypothesised the use of fibrin glue, a biological adhesive would work not only to allow adherence of the skin explants onto the scaffolds but be porous enough to allow cellular migration through the fibres.

Using concentrations of fibrinogen and thrombin (the two parent components of fibrin) from clinically used products in the literature we were able to derive a suitable form of fibrin that not only allowed the skin explants to adhere onto the scaffolds but did not hinder cellular migration and allowed for good tissue handling. This concentration of fibrinogen (18.75mg) and thrombin (2.5 I.U)

for use to adhere biological tissue to scaffolds was established in the MacNeil laboratory for adherence of limbal biopsies on scaffolds for corneal regeneration(164). This concentration when cultured with skin explants showed the most cellular outgrowth and allowed for handling of the skin explant pieces before the fibrin set into its gel like form.

We concluded that fibrin did make a significant contribution to skin explant retention onto the electrospun scaffolds which aided cellular migration and proliferation. Furthermore, this is clinically translatable as fibrin is FDA approved, however the concentration of the components of this fibrin doesn't exist commercially to the best of our knowledge.

8.5 Objective 4: The use of precultured scaffolds with fibroblasts as an in vivo wound bed model would help achieve better epithelial cell outgrowth from skin explants when used in combination with fibrin.

The use of skin explants to populate biodegradable electrospun scaffolds is a fairly new concept and as a result, the positive control to which it is compared is the traditional model of tissue engineered skin using acellular dermal matrix seeded with isolated keratinocytes and fibroblasts. Ultimately this scaffold material would be placed on a debrided wound bed from a full thickness wound, which will consist of an underlying vascular network surrounded by stroma, and other tissues eg subcutaneous fat or even fascia with a wound

edge. It was thought that pre culturing the scaffolds with fibroblasts, would allow them to proliferate and release growth factors and cytokines that would aid the migration of skin cells out of the skin explants, which would be much closer to what would happen in the in vivo setting on the wound bed. Quantitatively there was more cellular activity on these scaffolds more so when combined with fibrin, as expected. Furthermore, on confocal imaging pancytokeratin immunohistochemistry we were able to appreciate the migration of keratinocytes from the cut edge of the skin explants into the depths of our trilayer templated scaffolds. This further confirmed that skin explants are a viable source of cells that can provide a new epidermal cover on our synthetic scaffold, as indeed happens in Meek Grafting when keratinocytes and fibroblasts grow out of small pieces of split thickness graft onto the wound bed

One of the aims of this research was to avoid the need to provide laboratory expanded cultured cells in our constructs. In summary the presence of the fibroblasts did improve the outgrowth of cells from the explants suggesting that our main objective of using electrospun scaffolds combined with skin explants as an approach to treating full thickness skin defects remains a biological and clinical possibility.

Experimental Objectives	Main findings
1.The use of a trilayer PHBV/PGLA 75:25 electrospun scaffold versus monolayer PGLA 75:25 scaffolds	Mechanical properties of trilayer scaffolds were closer to that of skin Trilayer scaffolds handled better than monolayers Histologically there were definable layers in our constructs which mimicked the topography of skin
2.The use of metal templates to mimic the rete ridges of the epidermal-dermal interface	The results were unconvincing as there were no differences in cellular migration and proliferation when templated and non-templated scaffolds were used.
3.The use of fibrin as part of the skin explant/ electrospun scaffold model to secure the attachment of the skin explants to the electrospun scaffold	Fibrin that was made by using 18.75mg of fibrinogen and 2.5 I. U of thrombin was deemed to be the most appropriate form to allow skin explants to adhere onto the electrospun scaffold and at the same

	time not hinder cellular migration and proliferation.
4. The use of precultured electrospun scaffolds with fibroblasts provided a better wound bed model to mimic the in vivo microenvironment.	The use of this wound bed model confirmed the ability for skin explants to be cultured with fibrin on synthetic electrospun scaffolds to create tissue engineered skin without the need to use isolated cultured cells

Table 11: *Summary of experimental objectives and main findings*

8.6 Future Directions

The work in this thesis concentrated on assessing the suitability of using a trilayer electrospun scaffold for a neodermis, skin explants as a cell source and techniques to optimise the migration of skin cells from the skin explants onto the electrospun scaffolds. We learnt that it is possible, however we needed to define a few characteristics and use adjuncts to optimise this process. Although the use of trilayer scaffolds as a potential for dermal replacement has been previously been established in the MacNeil laboratory but with

laboratory cultured keratinocytes and fibroblasts(205), the use of skin explants as a cell source in tissue engineered skin is novel.

We found the skin explants needed to be further cut into small pieces to optimise spread with less dermis in skin explants facilitate more outward migration of epithelial cells and they can be orientated randomly. They need to stay in constant contact with the electrospun scaffolds to facilitate cellular migration and proliferation. To do this we used fibrin, a biological adhesive already in clinical use, with good results. Our in vitro studies have demonstrated that it is a possible to use biodegradable electrospun scaffolds combined with fibrin and skin explants to achieve cellular migration from the skin explants throughout the scaffold. The next step for this work would be do translate this onto animal models. Small full thickness wounds (to the subcutaneous layer) in the first instance would be created and reconstructed using our model. Scaffolds would be secured to the wound edges using a quick acting dissolving suture like vicryl rapide, commonly used to secure split skin grafts onto wounds. The skin explants would be laid onto the scaffolds with fibrin and dressed. The dressing in this instance is very important as it needs to facilitate the egress of fluid, the inflow of oxygen and not disturb the construct. One possibility is the use of a product akin to biobrane, which can be laid onto the top of the skin explants and scaffolds. This would be secured with tissue

glue and lifted off as epithelial cells populate the wound. This would also allow wound inspection for infection without disrupting the skin explants.

Once this work is completed then there are many more questions to be answered. Clinically important questions like, the depth of wound onto which is most appropriate eg muscle, tendon or bone. The maximal size of a wound that this would work in, the appearance clinically of what a regenerating wound using our model would look like and histological confirmation of the wound healing that has occurred. Other questions like the susceptibility to infection and which organisms in particular pose a potential threat to these scaffolds will need to be addressed. These points will all have to be defined as well as every step of the technique to allow for safe translation in using our trilayer electrospun/ skin explants into a 1st in man clinical safety study.

In conclusion this thesis has revealed that skin explants combined with fibrin and laid onto biodegradable synthetic trilayer electrospun scaffold result in cellular migration and proliferation that could facilitate healing in full thickness skin defects. These are early but important aspects of the groundwork that needs to occur so that in the future tissue engineered skin will be part of the routine armamentarium of the reconstructive surgeon.

References

1. Barker A. Skin structure. In: FRCS(Plast) RDFBs MBBS, MD, EBOPRASF, FRACS(Plast), Surgery NWBBs MBBS, MD, FRCS(Plast) Clinical Lead Plastic, Assistantessor SCM MSc, FRCSC, editors. Plastic and reconstructive surgery [Internet]. John Wiley & Sons, Ltd; 2015 [cited 2017 May 29]. p. 77–87. Available from: http://onlinelibrary.wiley.com/doi/10.1002/9781118655412.ch8/summary

2. Bhushan B, Tang W, Ge S. Nanomechanical characterization of skin and skin cream. J Microsc. 2010 Nov;240(2):135–44.

3. Proksch E, Brandner JM, Jensen J-M. The skin: an indispensable barrier. Exp Dermatol. 2008 Dec;17(12):1063–72.

4. Nishiyama T, Amano S, Tsunenaga M, Kadoya K, Takeda A, Adachi E, et al. The importance of laminin 5 in the dermal–epidermal basement membrane. J Dermatol Sci. 2000 Dec 1;24:S51–9.

5. Inoue S. Ultrastructure of Basement Membranes. Int Rev Cytol. 1989 Jan 1;117:57–98.

6. Ghalbzouri AE, Jonkman MF, Dijkman R, Ponec M. Basement Membrane Reconstruction in Human Skin Equivalents Is Regulated by Fibroblasts and/or Exogenously Activated Keratinocytes. J Invest Dermatol. 2005 Jan 1;124(1):79–86.

7. Kruegel J, Miosge N. Basement membrane components are key players in specialized extracellular matrices. Cell Mol Life Sci CMLS. 2010 Sep;67(17):2879–95.

8. Tsunenaga M, Adachi E, Amano S, Burgeson RE, Nishiyama T. Laminin 5 can promote assembly of the lamina densa in the skin equivalent model. Matrix Biol J Int Soc Matrix Biol. 1998 Dec;17(8–9):603–13.

9. Mathes S, Nahai F. Mathes SJ, Nahai F. Reconstructive Surgery: Principles, Anatomy & Technique. Vol. 2. New York: Churchill Livingstone; St. Louis: Quality Medical; 1997.; 1997.

10. Fawcet D, Jensh R. Concise Histology. New York: Chapman and Hall; 1997.

11. Odland GF. The morphology of the attachment between the dermis and the epidermis. Anat Rec. 1950 Nov;108(3):399–413.

12. Lavker R, Sun T. Heterogeneity in epidermal basal keratinocytes: morphological and functional correlations. Science. 1982;215:1239–41.

13. Jensen UB, Lowell S, Watt FM. The spatial relationship between stem cells and their progeny in the basal layer of human epidermis: a new view based on whole-mount labelling and lineage analysis. Dev Camb Engl. 1999 Jun;126(11):2409–18.

14.   Stern R, McPherson M, Longaker MT. Histologic study of artificial skin used in the treatment of full-thickness thermal injury. J Burn Care Rehabil. 1990 Feb;11(1):7–13.

15.   Santoro MM, Gaudino G. Cellular and molecular facets of keratinocyte reepithelization during wound healing. Exp Cell Res. 2005 Mar 10;304(1):274–86.

16.   Martin P. Wound healing--aiming for perfect skin regeneration. Science. 1997 Apr 4;276(5309):75–81.

17.   Sorrell JM, Caplan AI. Chapter 4 Fibroblasts—A Diverse Population at the Center of It All. Int Rev Cell Mol Biol. 2009 Jan 1;276:161–214.

18.   Weiss P. Cellular Dynamics. RevMod Phys. 1959;31:11–20.

19.   Grinnell F. Fibroblast biology in three-dimensional collagen matrices. Trends Cell Biol. 2003 May 1;13(5):264–9.

20.   Werner S, Krieg T, Smola H. Keratinocyte–Fibroblast Interactions in Wound Healing. J Invest Dermatol. 2007 May;127(5):998–1008.

21.   Hsu Y-C, Li L, Fuchs E. Transit-Amplifying Cells Orchestrate Stem Cell Activity and Tissue Regeneration. Cell. 2014 May 8;157(4):935–49.

22.   Zech NH, Preisegger K-H, Hollands P. Stem cell therapeutics—reality versus hype and hope. J Assist Reprod Genet. 2011 Apr;28(4):287–90.

23.   Blanpain C, Fuchs E. Epidermal Stem Cells of the Skin. Annu Rev Cell Dev Biol. 2006;22:339–73.

24.   Jones PH, Watt FM. Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression. Cell. 1993 May 21;73(4):713–24.

25.   Jones PH, Harper S, Watt FM. Stem cell patterning and fate in human epidermis. Cell. 1995 Jan 13;80(1):83–93.

26.   Li A, Simmons PJ, Kaur P. Identification and isolation of candidate human keratinocyte stem cells based on cell surface phenotype. Proc Natl Acad Sci U S A. 1998 Mar 31;95(7):3902–7.

27.   Ito M, Kizawa K. Expression of calcium-binding S100 proteins A4 and A6 in regions of the epithelial sac associated with the onset of hair follicle regeneration. J Invest Dermatol. 2001 Jun;116(6):956–63.

28.   Michel M, Török N, Godbout MJ, Lussier M, Gaudreau P, Royal A, et al. Keratin 19 as a biochemical marker of skin stem cells in vivo and in vitro: keratin 19 expressing cells are differentially localized in function of anatomic sites, and their number varies with donor age and culture stage. J Cell Sci. 1996 May;109 (Pt 5):1017–28.

29. Lyle S, Christofidou-Solomidou M, Liu Y, Elder DE, Albelda S, Cotsarelis G. The C8/144B monoclonal antibody recognizes cytokeratin 15 and defines the location of human hair follicle stem cells. J Cell Sci. 1998 Nov;111 (Pt 21):3179–88.

30. Trempus CS, Morris RJ, Bortner CD, Cotsarelis G, Faircloth RS, Reece JM, et al. Enrichment for living murine keratinocytes from the hair follicle bulge with the cell surface marker CD34. J Invest Dermatol. 2003 Apr;120(4):501–11.

31. Vangipuram M, Ting D, Kim S, Diaz R, Schüle B. Skin Punch Biopsy Explant Culture for Derivation of Primary Human Fibroblasts. J Vis Exp JoVE [Internet]. 2013 Jul 7 [cited 2017 Jun 25];(77). Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3731437/

32. Lee KC, Joory K, Moiemen NS. History of burns: The past, present and the future. Burns Trauma. 2014 Oct 25;2(4):169.

33. Klein M., Heimbach D, Gibran N. Management of the burn wound. New York: WebMD Inc; 2005.

34. Whittaker A. Treatment of burns by excision and immediate skin grafting. Am J Surg. 1953;85:411–7.

35. Jackson D. A clinical study of the use of skin homografts for burns. Br J Plast Surg. 1954 Jan 1;7:26–43.

36. Janzekovic ZMD. A NEW CONCEPT IN THE EARLY EXCISION AND IMMEDIATE GRAFTING OF BURNS. J Trauma-Inj Infect. 1970 Dec;10(12):1103–8.

37. Ong YS, Samuel M, Song C. Meta-analysis of early excision of burns. Burns J Int Soc Burn Inj. 2006 Mar;32(2):145–50.

38. Kansupada KB, Sassani JW. Sushruta: the father of Indian surgery and ophthalmology. Doc Ophthalmol Adv Ophthalmol. 1997;93(1–2):159–67.

39. EHRENFRIED A. Reverdin and Other Methods of Skin-Grafting. Boston Med Surg J. 1909 Dec 23;161(26):911–7.

40. Use of the Thiersch Skin Graft | The BMJ [Internet]. [cited 2017 May 21]. Available from: http://www.bmj.com/content/1/4142/846

41. Padgett EC. Indications for determination of the thickness for split skin grafts. Am J Surg. 1946 Nov 1;72(5):683–93.

42. Evans AJ. Use of the Humby Knife in the Excision of Burns. Br Med J. 1952 Nov 1;2(4791):979.

43. Fischl R. Skin grafting: a new technique. Br J Plast Surg. 1965;18(4):435–41.

44. Brown H. Motor driven dermatome. Ind Med Surg. 17(2):46.

45.   Meek C. Successful microdermagrafting using the Meek-wall microdermatome. Am J Surg. 96(4):557–8.

46.   Raff T, Hartmann B, Wagner H, Germann G. Experience with the modified Meek technique. Acta Chir Plast. 1996;38(4):142–6.

47.   Kreis R., Mackie D., Vloemans A. Widely expanded postage stamp skin grafts using a modified Meek technique in combination with an allograft overlay. Burns. 1993;19(2):142–5.

48.   Vandeput J, Tanner J., Carlisle J. The ultra postage stamp skin graft. Plast Reconstr Surg. 38(3):252–4.

49.   Hsieh C-S, Schuong J-Y, Huang WS, Huang TT. Five years' experience of the modified Meek technique in the management of extensive burns. Burns. 2008 May;34(3):350–4.

50.   Singh M, Nuutila K, Collins KC, Huang A. Evolution of skin grafting for treatment of burns: Reverdin pinch grafting to Tanner mesh grafting and beyond. Burns J Int Soc Burn Inj. 2017 Jan 30;

51.   Bargues L, Boyer S, Leclerc T, Duhamel P, Bey E. [Incidence and microbiology of infectious complications with the use of artificial skin Integra in burns]. Ann Chir Plast Esthet. 2009 Dec;54(6):533-9.

52.   Heimbach DM, Warden GD, Luterman A, Jordan MH, Ozobia N, Ryan CM, Voigt DW, Hickerson WL, Saffle JR, DeClement FA, Sheridan RL, Dimick AR. Multicenter postapproval clinical trial of Integra dermal regeneration template for burn treatment. J Burn Care Rehabil. 2003 Jan-Feb;24(1):42-8.

53.   Dunne JA, Murray A, Rawlins JM. A survey of skin substitute use in United Kingdom and Australasia. Burns J Int Soc Burn Inj. 2015 Mar;41(2):417.

54.   Girdner J. Skin Grafting with grafts taken from the dead subject. M Rec. 1881;20:119–20.

55.   Khoo TL, Halim AS, Saad AZM, Dorai AA. The application of glycerol-preserved skin allograft in the treatment of burn injuries: an analysis based on indications. Burns J Int Soc Burn Inj. 2010 Sep;36(6):897–904.

56.   Bromberg BE, Song IC. Homografts and heterografts as skin substitutes. Am J Surg. 1966 Jul;112(1):28–33.

57.   Shevchenko RV, James SL, James SE. A review of tissue-engineered skin bioconstructs available for skin reconstruction. J R Soc Interface. 2010 Feb 6;7(43):229–58.

58.   Saffle JR. Closure of the excised burn wound: temporary skin substitutes. Clin Plast Surg. 2009 Oct;36(4):627–41.

59. Narayan RP. Development of tissue bank. Indian J Plast Surg Off Publ Assoc Plast Surg India. 2012;45(2):396–402.

60. Backere AC. Euro Skin Bank : large scale skin-banking in Europe based on glycerol - preservation of donor skin. Burns. 1994;20(1):s4-d9.

61. RP Hermans, Hoekstra M, Kropman G, Koenderink J. The History and Function of the Euro Skin Bank. Ann Burns Fire Disasters. IX(1):1996.

62. Vardaxis NJ, Brans TA, Boon ME, Kreis RW, Marres LM. Confocal laser scanning microscopy of porcine skin: implications for human wound healing studies. J Anat. 1997 May;190 (Pt 4):601–11.

63. Armour AD, Fish JS, Woodhouse KA, Semple JL. A comparison of human and porcine acellularized dermis: interactions with human fibroblasts in vitro. Plast Reconstr Surg. 2006 Mar;117(3):845–56.

64. Feng X, Shen R, Tan J, Chen X, Pan Y, Ruan S, et al. The study of inhibiting systematic inflammatory response syndrome by applying xenogenic (porcine) acellular dermal matrix on second-degree burns. Burns J Int Soc Burn Inj. 2007 Jun;33(4):477–9.

65. May SR. The effects of biological wound dressings on the healing process. Clin Mater. 1991;8(3–4):243–9.

66. Raimer DW, Group AR, Petitt MS, Nosrati N, Yamazaki ML, Davis NA, et al. Porcine xenograft biosynthetic wound dressings for the management of postoperative Mohs wounds. Dermatol Online J. 2011 Sep 15;17(9):1.

67. Chern PL, Baum CL, Arpey CJ. Biologic dressings: current applications and limitations in dermatologic surgery. Dermatol Surg Off Publ Am Soc Dermatol Surg Al. 2009 Jun;35(6):891–906.

68. Heimbach D, Luterman A, Burke J, Cram A, Herndon D, Hunt J, et al. Artificial dermis for major burns. A multi-center randomized clinical trial. Ann Surg. 1988 Sep;208(3):313–20.

69. Lawin PB, Silverstein P, Still JM. E-Z Derm a porcine heterograft material. Am J Clin Dermatol. 2002;3(7):507; author reply 507-508.

70. Tavis MJ, Thornton JW, Bartlett RH, Roth JC, Woodroof EA. A new composite skin prosthesis. Burns. 1980 Nov 1;7(2):123–30.

71. Lal S, Barrow RE, Wolf S E. S. Lal, R.E. Barrow, S.E. Wolf, et al. Biobrane improves wound healing in burned children without increased risk of infection. Shock. 14(3):314–8.

72. Kucan J. Use of Biobrane in the treatment of toxic epidermal necrolysis. J Burn Care Rehabil. 16:324–57.

73. Bacha EA, Sheridan RL, Donohue GA, Tompkins RG. Staphylococcal toxic shock syndrome in a paediatric burn unit. Burns. 1994 Dec 1;20(6):499–502.

74. Weinzweig J, Gottlieb LJ, Krizek TJ. Toxic shock syndrome associated with use of biobrane in a scald burn victim. Burns. 1994 Apr 1;20(2):180–1.

75. Gallagher J, Williams-Bouyer N. Treatment of infection in burns. In: Total Burn Care. Third Edition. 2007.

76. Gallico GG, O'Connor NE, Compton CC, Kehinde O, Green H. Permanent coverage of large burn wounds with autologous cultured human epithelium. N Engl J Med. 1984 Aug 16;311(7):448–51.

77. Rue LW, Cioffi WG, McManus WF, Pruitt BA. Wound closure and outcome in extensively burned patients treated with cultured autologous keratinocytes. J Trauma. 1993 May;34(5):662–7; discussion 667-668.

78. Hernon CA, Dawson RA, Freedlander E, Short R, Haddow DB, Brotherston M, et al. Clinical experience using cultured epithelial autografts leads to an alternative methodology for transferring skin cells from the laboratory to the patient. Regen Med. 2006 Nov;1(6):809–21.

79. Navarro FA, Stoner ML, Park CS, Huertas JC, Lee HB, Wood FM, et al. Sprayed keratinocyte suspensions accelerate epidermal coverage in a porcine microwound model. J Burn Care Rehabil. 2000 Dec;21(6):513–8.

80. Harris P., Leigh H.A, Navsaria H. P.A. Harris, I.M. Leigh, H.A. Navsaria Pre-confluent keratinocyte grafting: the future for cultured skin replacements? Burns, 24 (1998), pp. 591-593. Burns. 1998;(24):591–3.

81. Stoner M, Wooe F. Stoner ML, Wood FM. The treatment of hypopigmented lesions with cultured epithelial autograft. J Burn Care Rehabil. 21(2000):50–4.

82. McAree KG, Klein RL, Boeckman CR. The use of cultured epithelial autografts in the wound care of severely burned patients. J Pediatr Surg. 1993 Feb 1;28(2):166–8.

83. Barret J., Wolf SE. Cost-efficacy of cultured epidermal autografts in massive pediatric burns. Ann Surg. 231(6):869–76.

84. Desai MH, Miakar J., McCauley K. Lack of long-term durability of cultured keratinocyte burn-wound coverage: a case report. J Burn Care Rehabil. 1991;12:540–5.

85. Meuli M, Raghunath M. Tops and flops using cultured epithelial autografts in children. Pediatr Surg Int. 1997;12:471–7.

86. O'Neill TB, Rawlins J, Rea S, Wood F. Treatment of a large congenital melanocytic nevus with dermabrasion and autologous cell suspension (ReCELL®): A case report. J Plast Reconstr Aesthet Surg. 2011 Dec 1;64(12):1672–6.

87.   Dunne JA, Saleh DB, Rawlins JM. Management of rhinophyma with Versajet™ and ReCell®. Br J Oral Maxillofac Surg. 2013 Dec;51(8):e282-284.

88.   Foster KN, Peck MD, Sood R, Feldman MJ, Jordan MH, Mozingo DW, Greenhalgh DG, Palmieri TL, Griswold JA, Dissanaike S, Hickerson WL. Holmes Iv JH, Molnar JA, Carter JE, Hwang J, Cairns BA, King BT, Smith DJ, Cruse CW, . A Comparative Study of the ReCell® Device and Autologous Spit-Thickness Meshed Skin Graft in the Treatment of Acute Burn Injuries. J Burn Care Res. 39(5):694–702.

89.   Gravante G, Di Fede MC, Araco A, Grimaldi M, De Angelis B, Arpino A, et al. A randomized trial comparing ReCell system of epidermal cells delivery versus classic skin grafts for the treatment of deep partial thickness burns. Burns J Int Soc Burn Inj. 2007 Dec;33(8):966–72.

90.   Wood F, Martin L, Lewis D, Rawlins J, McWilliams T, Burrows S, et al. A prospective randomised clinical pilot study to compare the effectiveness of Biobrane® synthetic wound dressing, with or without autologous cell suspension, to the local standard treatment regimen in paediatric scald injuries. Burns J Int Soc Burn Inj. 2012 Sep;38(6):830–9.

91.   National Insitutute for Healt and Care Excellence. The ReCell Spray-On Skin system for treating skin loss, scarring and depigmentation after burn injury. 2014 Nov. (Medical Technology guidance).

92.   Yannas IV, Burke JF. Design of an artificial skin. I. Basic design principles. J Biomed Mater Res. 1980 Jan 1;14(1):65–81.

93.   Yannas IV, Burke JF, Gordon PL, Huang C, Rubenstein RH. Design of an artificial skin. II. Control of chemical composition. J Biomed Mater Res. 1980 Mar;14(2):107–32.

94.   Jones I, Currie L, Martin R. A guide to biological skin substitutes. Br J Plast Surg. 2002 Apr;55(3):185–93.

95.   Burke JF, Yannas IV, Quinby WC, Bondoc CC, Jung WK. Successful use of a physiologically acceptable artificial skin in the treatment of extensive burn injury. Ann Surg. 1981 Oct;194(4):413–28.

96.   Sheridan RL, Hegarty M, Tompkins RG, Burke JF. Artificial skin in massive burns — results to ten years. Eur J Plast Surg. 1994 Mar 1;17(2):91–3.

97.   Min JH, Yun IS, Lew DH, Roh TS, Lee WJ. The Use of Matriderm and Autologous Skin Graft in the Treatment of Full Thickness Skin Defects. Arch Plast Surg. 2014 Jul;41(4):330–6.

98.   Ryssel H, Gazyakan E, Germann G, Öhlbauer M. The use of MatriDerm® in early excision and simultaneous autologous skin grafting in burns—A pilot study. Burns. 2008 Feb 1;34(1):93–7.

99.   Choi J-Y, Kim S-H, Oh G-J, Roh S-G, Lee N-H, Yang K-M. Management of Defects on Lower Extremities with the Use of Matriderm and Skin Graft. Arch Plast Surg. 2014 Jul;41(4):337–43.

100.   Boyce ST, Kagan RJ, Meyer NA, Yakuboff KP, Warden GD. The 1999 clinical research award. Cultured skin substitutes combined with Integra Artificial Skin to replace native skin autograft and allograft for the closure of excised full-thickness burns. J Burn Care Rehabil. 1999 Dec;20(6):453–61.

101.   Boyce ST, Hansbrough JF. Biologic attachment, growth, and differentiation of cultured human epidermal keratinocytes on a graftable collagen and chondroitin-6-sulfate substrate. Surgery. 1988 Apr;103(4):421–31.

102.   Boyce ST, Goretsky MJ, Greenhalgh DG, Kagan RJ, Rieman MT, Warden GD. Comparative assessment of cultured skin substitutes and native skin autograft for treatment of full-thickness burns. Ann Surg. 1995 Dec;222(6):743–52.

103.   Eaglstein WH, Iriondo M, Laszlo K. A composite skin substitute (graftskin) for surgical wounds. A clinical experience. Dermatol Surg Off Publ Am Soc Dermatol Surg Al. 1995 Oct;21(10):839–43.

104.   Zaulyanov L, Kirsner RS. A review of a bi-layered living cell treatment (Apligraf ®) in the treatment of venous leg ulcers and diabetic foot ulcers. Clin Interv Aging. 2007 Mar;2(1):93–8.

105.   Gohari S, Gambla C, Healey M, Spaulding G, Gordon KB, Swan J, et al. Evaluation of tissue-engineered skin (human skin substitute) and secondary intention healing in the treatment of full thickness wounds after Mohs micrographic or excisional surgery. Dermatol Surg Off Publ Am Soc Dermatol Surg Al. 2002 Dec;28(12):1107–14; discussion 1114.

106.   Muhart M, McFalls S, Kirsner RS, Elgart GW, Kerdel F, Sabolinski ML, et al. Behavior of tissue-engineered skin: a comparison of a living skin equivalent, autograft, and occlusive dressing in human donor sites. Arch Dermatol. 1999 Aug;135(8):913–8.

107.   Hayes DW, Webb GE, Mandracchia VJ, John KJ. Full-thickness burn of the foot: successful treatment with Apligraf. A case report. Clin Podiatr Med Surg. 2001 Jan;18(1):179–88.

108.   Davis SC, Martinez L, Kirsner R. The diabetic foot: the importance of biofilms and wound bed preparation. Curr Diab Rep. 2006 Dec;6(6):439–45.

109.   Cavorsi J, Vicari F, Wirthlin DJ, Ennis W, Kirsner R, O'Connell SM, et al. Best-practice algorithms for the use of a bilayered living cell therapy (Apligraf) in the treatment of lower-extremity ulcers. Wound Repair Regen Off Publ Wound Heal Soc Eur Tissue Repair Soc. 2006 Apr;14(2):102–9.

110. Mangır N, Bullock AJ, Roman S, Osman N, Chapple C, MacNeil S. Production of ascorbic acid releasing biomaterials for pelvic floor repair. Acta Biomater. 2016 Jan 1;29:188–97.

111. Cantón I, Mckean R, Charnley M, Blackwood KA, Fiorica C, Ryan AJ, et al. Development of an Ibuprofen-releasing biodegradable PLA/PGA electrospun scaffold for tissue regeneration. Biotechnol Bioeng. 2010 Feb 1;105(2):396–408.

112. Mangır N, Hilary C, Chapple C, Mac Neil S. Oestradiol-releasing Biodegradable Mesh Stimulates Collagen Production and Angiogenesis: An Approach to Improving Biomaterial Integration in Pelvic Floor Repair. E. Eur Urol Focus. 2017;

113. Doshi J, Reneker D. Electrospinning process and applications of electrospun fibers. Journal of electrostatics. 1995;35(2):151–60.

114. Bye FJ, Bissoli J, Black L, Bullock AJ, Puwanun S, Moharamzadeh K, et al. Development of bilayer and trilayer nanofibrous/microfibrous scaffolds for regenerative medicine. Biomater Sci. 2013 Jul 30;1(9):942–51.

115. Nair LS, Laurencin CT. Biodegradable polymers as biomaterials. Prog Polym Sci. 2007 Aug;32(8–9):762–98.

116. Hofmann GO. Biodegradable implants in orthopaedic surgery—A review on the state-of-the-art. Clin Mater. 1992 Jan 1;10(1):75–80.

117. Long-term (>10 years) lactic acid coronary stents: Igaki-Tamai stents.

118. Coronary stents: historical development, current status and future directions | British Medical Bulletin | Oxford Academic [Internet]. [cited 2017 Jan 29]. Available from: https://academic.oup.com/bmb/article-lookup/doi/10.1093/bmb/ldt009

119. Kapoor DN, Bhatia A, Kaur R, Sharma R, Kaur G, Dhawan S. PLGA: a unique polymer for drug delivery. Ther Deliv. 2015 Jan 1;6(1):41–58.

120. Biodegradable polymers as biomaterials [Internet]. [cited 2017 Jan 29]. Available from: http://www.sciencedirect.com/science/article/pii/S0079670007000664?np=y&npKey =6cffcc29eaeaeb9c22a4c5f4794d6ea18c122dfbdfc3d62eaaa220e8f281c7a1

121. Maitz MF. Applications of synthetic polymers in clinical medicine. Biosurface Biotribology. 2015 Sep;1(3):161–76.

122. Mooney DJ, Mazzoni CL, Breuer C, McNamara K, Hern D, Vacanti JP, et al. Stabilized polyglycolic acid fibre-based tubes for tissue engineering. Biomaterials. 1996 Jan;17(2):115–24.

123. Li S. Hydrolytic degradation characteristics of aliphatic polyesters derived from lactic and glycolic acids. J Biomed Mater Res. 1999 Jan 1;48(3):342–53.

124. Blackwood KA, McKean R, Canton I, Freeman CO, Franklin KL, Cole D, et al. Development of biodegradable electrospun scaffolds for dermal replacement. Biomaterials. 2008 Jul;29(21):3091–104.

125. McKenna KA, Hinds MT, Sarao RC, Wu P-C, Maslen CL, Glanville RW, et al. Mechanical Property Characterization of Electrospun Recombinant Human Tropoelastin for Vascular Graft Biomaterials. Acta Biomater. 2012 Jan;8(1):225–33.

126. Williams JM, Adewunmi A, Schek RM, Flanagan CL, Krebsbach PH, Feinberg SE, et al. Bone tissue engineering using polycaprolactone scaffolds fabricated via selective laser sintering. Biomaterials. 2005 Aug;26(23):4817–27.

127. Escoffier C, de Rigal J, Rochefort A, Vasselet R, Lévêque JL, Agache PG. Age-related mechanical properties of human skin: an in vivo study. J Invest Dermatol. 1989 Sep;93(3):353-7.

128. Boudriot U, Dersch R, Greiner A, Wendorff JH. Electrospinning Approaches Toward Scaffold Engineering—A Brief Overview. Artif Organs. 2006 Oct 1;30(10):785–92.

129. Li W-J, Laurencin CT, Caterson EJ, Tuan RS, Ko FK. Electrospun nanofibrous structure: A novel scaffold for tissue engineering. J Biomed Mater Res. 2002 Jun 15;60(4):613–21.

130. Vaquette C, Cooper-White JJ. Increasing electrospun scaffold pore size with tailored collectors for improved cell penetration. Acta Biomater. 2011 Jun;7(6):2544–57.

131. Zhang D, Chang J. Patterning of Electrospun Fibers Using Electroconductive Templates. Adv Mater. 2007 Nov 5;19(21):3664–7.

132. Basu S, Sureka SP, Shanbhag SS, Kethiri AR, Singh V, Sangwan VS. Simple Limbal Epithelial Transplantation: Long-Term Clinical Outcomes in 125 Cases of Unilateral Chronic Ocular Surface Burns. Ophthalmology. 2016 May;123(5):1000–10.

133. Ortega I, McKean R, J. Ryan A, MacNeil S, Claeyssens F. Characterisation and evaluation of the impact of microfabricated pockets on the performance of limbal epithelial stem cells in biodegradable PLGA membranes for corneal regeneration. Biomater Sci. 2014;2(5):723–34.

134. Lovett M, Lee K, Edwards A, Kaplan DL. Vascularization Strategies for Tissue Engineering. Tissue Eng Part B Rev. 2009 Sep;15(3):353–70.

135. Braghirolli DI, Helfer VE, Chagastelles PC, Dalberto TP, Gamba D, Pranke P. Electrospun scaffolds functionalized with heparin and vascular endothelial growth factor increase the proliferation of endothelial progenitor cells. Biomed Mater. 2017;12(2):025003.

136. Gigliobianco G, Chong CK, MacNeil S. Simple surface coating of electrospun poly-L-lactic acid scaffolds to induce angiogenesis. J Biomater Appl. 2015 Jul 1;30(1):50–60.

137. Barnes MJ. Function of Ascorbic Acid in Collagen Metabolism. Ann N Y Acad Sci. 1975 Sep 1;258(1):264–77.

138. Kumbar SG. Electrospun poly(lactic acid-co-glycolic acid) scaffolds for skin tissue engineering. Biomaterials. 2008 Oct 1;29(30):4100–7.

139. Bhowmick S, Rother, Sandra. Biomimetic electrospun scaffolds from main extracellular matrix components for skin tissue engineering application – The role of chondroitin sulfate and sulfated hyaluronan. Mater Sci Eng C. 2017 Oct 1;79:15–22.

140. Sadeghi A, Mahdizadeh A. Surface modification of electrospun PLGA scaffold with collagen for bioengineered skin substitutes. Mater Sci Eng C. 2016 Sep 1;66:130–7.

141. Venugopal JR, Zhang Y, Ramakrishna S. In vitro culture of human dermal fibroblasts on electrospun polycaprolactone collagen nanofibrous membrane. Artif Organs. 2006 Jun;30(6):440–6.

142. Chen H, Huang J, Yu J, Liu S, Gu P. Electrospun chitosan-graft-poly (ε -caprolactone)/poly (ε-caprolactone) cationic nanofibrous mats as potential scaffolds for skin tissue engineering. Int J Biol Macromol. 2011 Jan 1;48(1):13–9.

143. Blombäck B. FIBRINOGEN AND FIBRIN-PROTEINS WITH COMPLEX ROLES IN HEMOSTASIS AND THROMBOSIS. Thromb Res. 1996 Jul 1;83(1):1–75.

144. Ronfard V, Huart J. Use of human keratinocytes cultured on fibrin glue in the treatment of burn wounds. Burns. 1991;17(3):181–4.

145. Gorodetsky R, Marx G. Fibrin Microbeads (FMB) as Biodegradable Carriers for Culturing Cells and for Accelerating Wound Healing. J Invest Dermatol. 112(6):866–72.

146. Krasna M, Jeras M. Evaluation of a fibrin-based skin substitute prepared in a defined keratinocyte medium,. IntJPharm. 2005;291(1–2):31–7.

147. clark RA., Colvin R. Fibronectin and Fibrin Provide a Provisional Matrix for Epidermal Cell Migration During Wound Reepithelialization, J.Invest.Dermatol. 79(5), 264-269 (1982). JInvest.dermatol. 1982;79(5):264–9.

148. Ahmed TAE, Dare EV, Hincke M. Fibrin: a versatile scaffold for tissue engineering applications. Tissue Eng Part B Rev. 2008 Jun;14(2):199–215.

149. Good medical practice (2013) [Internet]. [cited 2017 May 2]. Available from: http://www.gmc-uk.org/guidance/good_medical_practice.asp

150. Human Tissue Act 2004 | Human Tissue Authority [Internet]. [cited 2017 Jun 17]. Available from: https://www.hta.gov.uk/policies/human-tissue-act-2004

151. Gunatillake PA, Adhikari R. Biodegradable synthetic polymers for tissue engineering. Eur Cell Mater. 2003 May 20;5:1–16; discussion 16.

152. Tsuji H, Ikarashi K. In vitro hydrolysis of poly(l-lactide) crystalline residues as extended-chain crystallites. Part I: long-term hydrolysis in phosphate-buffered solution at 37°C. Biomaterials. 2004 Nov;25(24):5449–55.

153. Luzier WD. Materials derived from biomass/biodegradable materials. Proc Natl Acad Sci. 1992 Feb 1;89(3):839–42.

154. Yasin M, Holland SJ, Tighe BJ. Polymers for biodegradable medical devices. V. Hydroxybutyrate-hydroxyvalerate copolymers: effects of polymer processing on hydrolytic degradation. Biomaterials. 1990 Sep;11(7):451–4.

155. Holland SJ, Jolly AM, Yasin M, Tighe BJ. Polymers for biodegradable medical devices. II. Hydroxybutyrate-hydroxyvalerate copolymers: hydrolytic degradation studies. Biomaterials. 1987 Jul;8(4):289–95.

156. Chan BP, Leong KW. Scaffolding in tissue engineering: general approaches and tissue-specific considerations. Eur Spine J. 2008 Dec;17(Suppl 4):467–79.

157. O'Brien FJ. Biomaterials & scaffolds for tissue engineering. Mater Today. 2011 Mar;14(3):88–95.

158. Babensee JE, Anderson JM, McIntire LV, Mikos AG. Host response to tissue engineered devices. Adv Drug Deliv Rev. 1998 Aug 3;33(1):111–39.

159. Geerligs, M, Van Breemen, L, Peters, G. In vitro indentation to determine the mechanical properties of epidermis. J Biomech 2011; 44: 1176–1181.

160. Crichton, ML, Donose, BC, Chen, X. The viscoelastic, hyperelastic and scale dependent behaviour of freshly excised individual skin layers. Biomaterials 2011; 32: 4670–4681.

161. Hutmacher DW. Scaffolds in tissue engineering bone and cartilage. Biomaterials. 2000 Dec 15;21(24):2529–43.

162. Schmidt DR, Waldeck H, Kao WJ. Protein Adsorption to Biomaterials. In: Puleo DA, Bizios R, editors. Biological Interactions on Materials Surfaces [Internet]. Springer US; 2009 [cited 2017 May 25]. p. 1–18. Available from: http://link.springer.com/chapter/10.1007/978-0-387-98161-1_1

163. Partap S, Lyons F, O'Brien FJ. IV.1. Scaffolds & surfaces. Stud Health Technol Inform. 2010;152:187–201.

164. Ortega Í, Sefat F, Deshpande P, Paterson T, Ramachandran C, Ryan AJ, et al. Combination of Microstereolithography and Electrospinning to Produce Membranes Equipped with Niches for Corneal Regeneration. JoVE J Vis Exp. 2014 Sep 12;(91):e51826–e51826.

165. Dixit S, Baganizi D, Sahu R. Immunological challenges associated with artificial skin grafts: available solutions and stem cells in future design of synthetic skin. J Biol Eng. 2017(11):49.

166. Hedlin P, Taschuk R, Potter A, Griebel P, Napper S. Detection and Control of Prion Diseases in Food Animals. ISRN Vet Sci. 2012;2012.

167. Badylak SF. The extracellular matrix as a scaffold for tissue reconstruction. Semin Cell Dev Biol. 2002 Oct;13(5):377–83.

168. Penrose L, Ohara P. The development of the epidermal ridges. J Med Genet. 1973;10(3):201–8.

169. McCullough J, Kelly K. Prevention and treatment of skin aging. Ann N Acad Sci. 2006;1067:323–31.

170. Giangreco A, Goldie A, Failla A. Human skin aging is associated with reduced expression of the stem cell markers beta1 integrin and MCSP. J Invest Dermatol. 2010;130(2):604–8.

171. Chester DL,Balderson DS, Papini RP. A review of keratinocyte delivery to the wound bed. J Burn Care Rehabil. 2004 May-Jun;25(3):266-75. J Burn Care Rehabil. 2004;25(3):266–75.

172. Y. Poumay, M.R. Pittelkow. Cell density and culture factors regulate keratinocyte commitment to differentiation and expression of suprabasal K1/K10 keratins. J Invest Dermatol. 1995;104:271–6.

173. Ortega Í, Ryan AJ, Deshpande P, MacNeil S, Claeyssens F. Combined microfabrication and electrospinning to produce 3-D architectures for corneal repair. Acta Biomater. 2013 Mar;9(3):5511–20.

174. Estrach S, Legg J, Watt F. Syntenin mediates Delta1-induced cohesiveness of epidermal stem cells in culture. J Cell Sci. 2007;120:2944–52.

175. Chong EJ, Phan TT, Lim IJ, Zhang YZ, Bay BH, Ramakrishna S, et al. Evaluation of electrospun PCL/gelatin nanofibrous scaffold for wound healing and layered dermal reconstitution. Acta Biomater. 2007 May;3(3):321–30.

176. Guo H-F, Li Z-S, Dong S-W, Chen W-J, Deng L, Wang Y-F, et al. Piezoelectric PU/PVDF electrospun scaffolds for wound healing applications. Colloids Surf B Biointerfaces. 2012 Aug 1;96:29–36.

177. Chandrasekaran AR, Venugopal J, Sundarrajan S, Ramakrishna S. Fabrication of a nanofibrous scaffold with improved bioactivity for culture of human dermal fibroblasts for skin regeneration. Biomed Mater Bristol Engl. 2011 Feb;6(1):015001.

178. Lin H, Chen S, Chang S, Huang ST. Lin HY, Chen SH, Chang SH, Huang ST. Tri-layered chitosan scaffold as a potential skin substitute. J Biomater Sci Polym Ed. 2015;26(13):855–67.

179. Paterson TE, Beal SN, Santocildes-Romero ME, Sidambe AT, Hatton PV, Asencio. Selective laser melting-enabled electrospinning: Introducing complexity within electrospun membranes. Proc Inst Mech Eng H. 2017;231(6):565–74.

180. Choi S, Zhang Y, Macewan M. Neovascularization in biodegradable inverse opal scaffolds with uniform and precisely controlled pore sizes. Adv Heal Mater. 2013;2:145–54.

181. Debels H, Hamdi M, Abberton K. Dermal matri- ces and bioengineered skin substitutes: a critical review of current options. Plast Reconstr Surg Glob Open. 2015(3):e284.

182. Ring A, Langer S, Schaffran A. Enhanced neovas- cularization of dermis substitutes via low-pressure plasma-mediated surface activation. Burns. 36:1222–7.

183. Scherer S, Pietramaggiori G, Matthews J. Poly- N-acetyl glucosamine nanofibers: a new bioactive material to enhance diabetic wound healing by cell migration and angiogenesis. Ann Surg. 250:322–30.

184. Langer R. Drug delivery and targeting. Nature. 1998;392:5–10.

185. Dew L, English WR, Ortega I, Claeyssens F, MacNeil S. Fabrication of Biodegradable Synthetic Vascular Networks and Their Use as a Model of Angiogenesis. Cells Tissues Organs. 2016;202(5–6):319–28.

186. Li W, Lan Y, Guo R. In vitro and in vivo evalu- ation of a novel collagen/cellulose nanocrystals scaf- fold for achieving the sustained release of basic fibroblast growth factor. J Biomater Appl. 2015b;29:882–93.

187. Liu Q, Huang Y, Lan Y, Zuo Q. Acceleration of skin regeneration in full-thickness burns by incorporation of bFGF-loaded alginate microspheres into a CMCS-PVA hydrogel. J Tissue Eng Regen Med. 2017;11(5):1562–73.

188. Muhammad Yar, Lubna Shahzadi, Azra Mehmood, Muhammad Imran Raheem, Sabiniano Román, Aqif Anwar Chaudhry, Ihtesham ur Rehman, C.W. Ian Douglas, Sheila MacNeil,. Deoxy-sugar releasing biodegradable hydrogels promote angiogenesis and stimulate wound healing, Materials Today Communications, Volume 13, 2017, Pages 295-305,. Mater Today Commun. 2017;13:295–305.

189. Montano I, Schiestl C, Schneider J. Formation of human capillaries in vitro: the engineering of prevascularized matrices. Tissue Eng Part A. 2010;16:269–82.

190. Supp D, Wilson-Landy K, Boyce S. Human dermal microvascular endothelial cells form vascular analogs in cultured skin substitutes after grafting to athymic mice. FASEB J. 2002;(16):797–804.

191. Athanassopoulous A, Tsaknakis G, Newey S. Microvessel networks pre-formed in artifi- cial clinical grade dermal substitutes in vitro using cells from haematopoietic tissues. Burns. 38:691–701.

192. Laschke M, Giebels C, Menger M. Vasculogenesis: a new piece of the endometriosis puzzle. Hum Reprod Update. 2011;17:628–36.

193. Markowicz M, E Koellensperger, Neuss S. Human bone marrow mesenchymal stem cells seeded on modified collagen improved dermal regeneration in vivo. Cell Transpl. 2006;(15):723–32.

194. Liu P, Deng Z, Han S. Tissue-engineered skin containing mesenchymal stem cells improves burn wounds. Artif Organs. 2008;32:925–31.

195. Shahzadi L, Yar M, Jamal A. Triethyl orthoformate covalently cross-linked chitosan-(poly vinyl) alcohol based biodegradable scaffolds with heparin-binding ability for promoting neovascularisation. J Biomater Appl. 2016;31(4):582–93.

196. Hudson BG, Reeders ST, Tryggvason K. Type IV collagen: structure, gene organization, and role in human diseases. Molecular basis of Goodpasture and Alport syndromes and diffuse leiomyomatosis. J Biol Chem. 1993 Dec 15;268(35):26033–6.

197. Kühn K. Basement membrane (type IV) collagen. Matrix Biol J Int Soc Matrix Biol. 1995 Feb;14(6):439–45.

198. Timpl R. Proteoglycans of basement membranes. Experientia. 1993 May 15;49(5):417–28.

199. Timpl R, Brown JC. Supramolecular assembly of basement membranes. BioEssays News Rev Mol Cell Dev Biol. 1996 Feb;18(2):123–32.

200. Bonewald LF. Regulation and regulatory activities of transforming growth factor beta. Crit Rev Eukaryot Gene Expr. 1999;9(1):33–44.

201. Smola H, Stark HJ, Thiekötter G, Mirancea N, Krieg T, Fusenig NE. Dynamics of basement membrane formation by keratinocyte-fibroblast interactions in organotypic skin culture. Exp Cell Res. 1998 Mar 15;239(2):399–410.

202. Varkey M, Ding J, Tredget EE. Advances in Skin Substitutes—Potential of Tissue Engineered Skin for Facilitating Anti-Fibrotic Healing. J Funct Biomater. 2015 Jul 9;6(3):547–63.

203. Jha BS, Ayres CE, Bowman JR, Telemeco TA, Sell SA, Bowlin GL, et al. Electrospun Collagen: A Tissue Engineering Scaffold with Unique Functional Properties in a Wide Variety of Applications [Internet]. Journal of Nanomaterials. 2011 [cited 2017 Jul 2]. Available from: https://www.hindawi.com/journals/jnm/2011/348268/

204. Bridge JC, Aylott JW, Brightling CE, Ghaemmaghami AM, Knox AJ, Lewis MP, et al. Adapting the Electrospinning Process to Provide Three Unique Environments for a Tri-layered In Vitro Model of the Airway Wall. J Vis Exp JoVE [Internet]. 2015 Jul 31 [cited 2017 Jul 2];(101). Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4544510/

205. Bye FJ, Bullock AJ, Singh R, Sefat F, Roman S, MacNeil S. Development of a Basement Membrane Substitute Incorporated Into an Electrospun Scaffold for 3D Skin Tissue Engineering. J Biomater Tissue Eng. 2014 Sep 1;4(9):686–92.

206. Powell HM, Boyce ST. Engineered human skin fabricated using electrospun collagen-PCL blends: morphogenesis and mechanical properties. Tissue Eng Part A. 2009 Aug;15(8):2177–87.

207. Thomas V, Jagani S, Johnson K, Jose MV, Dean DR, Vohra YK, et al. Electrospun Bioactive Nanocomposite Scaffolds of Polycaprolactone and Nanohydroxyapatite for Bone Tissue Engineering. J Nanosci Nanotechnol. 2006 Feb 1;6(2):487–93.

208. Sood R, Roggy D, Zieger M, Balledux J, Chaudhari S, Koumanis DJ, et al. Cultured epithelial autografts for coverage of large burn wounds in eighty-eight patients: the Indiana University experience. J Burn Care Res Off Publ Am Burn Assoc. 2010 Aug;31(4):559–68.

209. Clugston P, Snelling C, MacDonald I. Cultured epithelial autografts: three years of clinical experience with eighteen patients. J Burn Care Rehabil. 12(6):533–9.

210. Cheng F, Shen Y, Mohanasundaram P, Lindström M, Ivaska J, Ny T, et al. Vimentin coordinates fibroblast proliferation and keratinocyte differentiation in wound healing via TGF-β–Slug signaling. Proc Natl Acad Sci U S A. 2016 Jul 26;113(30):E4320–7.

211. Rheinwald, Green. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell. 1975;6:331–43.

212. Boxman C, Ruwhof O., Boerman C. Role of fibroblasts in the regulation of pro-inflammatory interleukin IL-1, IL-6 and IL-8 levels induced by keratinocyte-derived IL-1. Arch Dermatol Res. 1996;288:391–8.

213. Scherer-Pietramaggiori SS, Pietramaggiori G, Orgill D. Plastic Surgery: Volume ONe: Principles, Skin Graft. 3rd ed. Seattle: Elsevier; 2013. (Neligan's Plastic surgery; vol. One).

214. Janmey P, Winer J, Weisel J. Fibrin gels and their clinical and bioengineering applications. J R Soc Interface. 2009;6:1–10.

215. Olfat G, Egles C, Bencherif S. Fibrin: An underrated biopolymer for skin tissue engineering. J Mol Biol Ad Biotechnol.

216. Laurens N, Koolwijk P, De Maat M. Fibrin structure and wound healing. J ThrombHaemost 4: 932-939. ThrombHaemost. 2006;4(932–939).

217. Blomback B, Carlsson K, Fatah B. Fibrin in human plasma: gel architectures governed by rate and nature of fibrinogen activation. Thromb Res. 1994;75:521–38.

218. Wolberg AS. Thrombin generation and fibrin clot structure. Blood Rev. 2007 May 1;21(3):131–42.

219. Alston SM, Solen KA, Broderick AH, Sukavaneshvar S, Mohammad SF. New method to prepare autologous fibrin glue on demand. Transl Res J Lab Clin Med. 2007 Apr;149(4):187–95.

220. Baxter. Artiss [Internet]. Available from: https://www.fda.gov/downloads/biologicsbloodvaccines/bloodbloodproducts/approvedproducts/licensedproductsblas/fractionatedplasmaproducts/ucm073054.pdf

221. Baxter. Tisseel [Internet]. Baxter; Available from: BloodProducts/ApprovedProducts/LicensedProductsBLAs/FractionatedPlasmaProducts/ucm072968.pdf

222. Feenstra RP, Tseng SC. Comparison of fluorescein and rose bengal staining. Ophthalmology. 1992 Apr;99(4):605–17.

223. Boyce ST, Warden GD. Principles and practices for treatment of cutaneous wounds with cultured skin substitutes. Am J Surg. 2002 Apr 1;183(4):445–56.

224. Spotnitz WD. Efficacy and safety of fibrin sealant for tissue adherence in facial rhytidectomy. Clin Cosmet Investig Dermatol. 2012 May 24;5:43–51.

225. Wong VW, Levi B, Rajadas J, Longaker MT, Gurtner GC. Stem Cell Niches for Skin Regeneration. Int J Biomater. 2012 Jun 3;2012:e926059.

226. Järveläinen H, Sainio A, Koulu M, Wight TN, Penttinen R. Extracellular matrix molecules: potential targets in pharmacotherapy. Pharmacol Rev. 2009;61(2):198–223.

227. WEINSTEIN GD, BOUCEK RJ. Collagen and elastin of human dermis. J Invest Dermatol. 1960;35:227–9.

228. Okamoto O, Fujiwara S. Dermatopontin, a novel player in the biology of the extracellular matrix. Connect Tissue Res. 2006;47(4):177–89.

229. Bainbridge P. Wound healing and the role of fibroblasts. Wound Care. 2013;22(8):407–8.

230. Lamme EN, Van Leeuwen RT, Brandsma K, Van Marle J, Middelkoop E. Higher numbers of autologous fibroblasts in an artificial dermal substitute improve tissue regeneration and modulate scar tissue formation. J Pathol. 2000;190(5):595–603.

231. Huang YC, Wang TW, Sun JS, Lin FH. Cultured keratinocytes and dermal fibroblasts on a double-layer scaffold with bi-medium culture system. Biomed Sci Instrum. 2003;39:500-5. Biomed Sci Instrum. 2003;39:500–5.

232. Dai NT, Yeh MK, Liu DD, Adams EF, Chiang CH, Yen CY, Shih CM, Sytwu HK, Chen, TM, Wang HJ, Williamson MR, Coombes AG. A co-cultured skin model based on cell support membranes. Biochem Biophys Res Commun. 2005;329(3):905–8.

233. Sobecki M, Mrouj K, Camasses A, et al. The cell proliferation antigen Ki-67 organises heterochromatin. Watt FM, ed. eLife. 2016;5:e13722. doi:10.7554/eLife.13722. eLife. 2016;5:e13722.

234. Lavker RM, Sun TT. Heterogeneity in epidermal basal keratinocytes: morphological and functional correlations. Science. 1982 Mar 5;215(4537):1239–41.

235. WHO. Burns. Available at http://www.who.int/mediacentre/factsheets/fs365/en/. Accessed February 27, 2016.

236. WHO. World report on disability. Available at http://www.who.int/disabilities/world_report/2011/en/. Accessed September 26, 2015.

Appendix 1: Consent forms and patient information leaflet for excess skin collection.

Consent form for using samples of human skin for research

- Thank you for reading the information about our research.
- If you would like to take part, please read and sign this form,
  a copy of which will be retained in your medical records.

Centre:
Department of Plastic Surgery, Sheffield Teaching Hospitals Trust

Title of project: Development of tissue engineered wound models for wound healing research for patients with burns and chronic wounds.

Name of Researcher: Professor Sheila MacNeil
Contact details for research team: Professor Sheila MacNeil, Biomaterials &Tissue Engineering Group, University of Sheffield, Kroto Research Institute, North Campus, Broad Lane, Sheffield, S3 7HQ.
Tel: +00 44 (114) 222 5995

Please Initial in the box

1. I have read the attached information sheet for this project, (version 3 dated 13/04/2016) and have been given a copy to keep. I have been able to ask questions about the project and I understand why the research is being done and any risks involved.

2. I do understand that some cells from the samples may be used in animal work.

3. I agree to give a sample of skin for research in this project. I understand how the sample will be collected, and that giving a sample for this research is voluntary.

4. I understand that I will not benefit financially, or otherwise, if this research leads to the development of a new treatment or medical test.

5. I know how to contact the research team if I need to, and how to get information about the results of the research.

6. Consent for storage and use in possible future research projects.
I agree that the sample I have given can be stored at the University of Sheffield for possible use in future projects to tackle some of problems which occur following burns injuries as described in the attached information sheet.

All donated skin samples will be stored anonymously and used only for laboratory research to benefit patients supervised by Professor MacNeil. All skin samples will be destroyed at the end of the experiments.

………………………………………… ……………………………… …………………………………………
Name of patient Date Signature
(BLOCK CAPITALS)

………………………………………… ……………………………… …………………………………………
Name of Surgeon taking consent Date Signature
(BLOCK CAPITALS)

Thank you for agreeing to participate in this research

Professor Sheila MacNeil
Professor of Biomaterials &Tissue Engineering
Kroto Research Institute
University of Sheffield
Email: s.macneil@sheffield.ac.uk

Dated 13th April 2016 Version 7
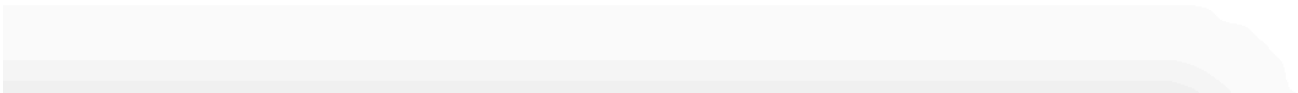
PATIENT INFORMATION SHEET

Donating clinically surplus human skin for laboratory based research

Sheffield Teaching Hospitals **NHS**
NHS Foundation Trust

Professor Sheila MacNeil
Tissue Engineering Group
KrotoResearch Institute
North Campus
University of Sheffield
Broad Lane
Sheffield
S3 7HQ
South Yorkshire
UK
Tel: +00 (44) 114 222 5995

Project title
Development of tissue engineered wound models for wound healing research for patients with burns and chronic wounds.

We would like you to consider this research study and then decide whether you would like to take part. Before you decide whether to participate or not it is important to understand why the research is being done and what it involves. Please read the following information carefully and decide whether you wish to take part.

1. Background Information on the project
Over the last 20 years or so, my research group has been developing a skin substitute for patients with burns or chronic wounds. Through our research we hope to able to offer this skin substitute to patients with severe burns especially to those who do not have enough of their own skin to be used for skin grafts. We also use this skin substitute to simulate the behaviour of normal skin under laboratory conditions.

The laboratory-based component of this research involves obtaining skin from patients such as yourself, who are having skin removed, and isolating and growing skin and stem cells from these samples. We also sterilise samples of skin to remove all the cells from them and use these sterilised samples to make new skin. If you agree to donate your skin, it will only be used in the laboratory to help us with our research. It will not be used on any patients. Depending on the research some cells from the samples may be implanted into animals.

2. Why have I been invited?
You are about to or have recently undergone surgery (abdominal reduction, breast reductions or body lift) that involves the removal of excess skin from your body. The removed skin would normally be discarded at the end of the operation. However, I would like to ask you whether you would be willing to donate the removed skin to our research laboratories at the Kroto Research Institute, University of Sheffield.

3. Will I need to go through extra tests?
You will not be required to undergo any extra tests or procedures as a result of donating your skin, and no extra skin will be removed as a result of your participation in this study.

All patients undergoing breast, abdominal reduction surgery or body lift will be approached regarding the donation of their skin to our research laboratory. However, it is up to you to decide whether you would like to donate your skin for this purpose. If you do decide to donate your skin, you will be given this information sheet to keep for reference. If you decide not to donate your skin then your treatment will not be affected in any way.

Please take time to read the following information and discuss it with others if you wish. Ask, if there is anything that is not clear, or if you would like more information. Take time to decide whether or not you wish to donate the skin that will be removed in your operation.

4. Skin donation

If you donate your skin, it will be treated as a freely donated gift from you to the Tissue Engineering Group. However, the knowledge gained from the gift of skin samples such as yours will hopefully benefit many patients in the future.

Once you have donated your skin sample, it will be given an anonymous reference number by the research group. This reference number and the date of your operation will be the only record they will have. Your sample will be completely anonymous. No other personal details about you are kept on record. The skin will be used for experiments within the Tissue Engineering Group, University of Sheffield and will be stored in the Kroto Research Institute Laboratories, Sheffield. Once the experiments using your skin sample have been completed, it will be disposed of properly, which in most cases is by incineration.

My research group will only use your skin to isolate cells and to make new skin in the laboratory. We will not do any diagnostic tests on the skin that have any relevance to your current or future health.

5. Can I change my mind?

Please note that you can change your mind about whether you wish to donate tissue for research or not, at any time prior to surgery. If we ask for consent after your surgery is finished and you are unsure about donating please do not consent. After surgery has taken place and the tissue is in the laboratory, it is not possible to return the tissue as all tissue for research is handled on an anonymous basis to protect your identity. We would therefore be unable to trace your donated tissue.

6. Will the research be published?

My research group will aim to publish the results of our research in medical journals and present the results at national and international conferences related to burns, chronic wound, plastic surgery and tissue engineering. If you would like to receive details and scientific results of our research, please write to me at the address on the front of this information sheet stating that you had donated your skin to us during your surgery.

Thank you for taking the time to read this information sheet.

Chief Investigator
Professor Sheila MacNeil
Professor of Biomaterials &Tissue Engineering
Kroto Research Institute
University of Sheffield
Email: s.macneil@sheffield.ac.uk

Appendix 2: Ethical approval for the collection of human skin

NRES Committee Yorkshire & The Humber - Sheffield

Jarrow Business Centre
Viking Business Park
Rolling Mill Road
Jarrow
Tyne and Wear
NE32 3DT

Telephone: 0191 4283564

03 June 2015

Prof Sheila MacNeil
Kroto Research Institute
University of Sheffield
Broad Lane, Sheffield
S3 7HQ

Dear Prof MacNeil

Title of the Research Tissue Bank:	**Research Tissue Bank**
REC reference:	**15/YH/0177**
Designated Individual:	**Prof Sheila MacNeil**
IRAS project ID:	**128409**

Thank you for your letter of 21 May 2015, responding to the Committee's request for further information on the above research tissue bank and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this favourable opinion letter. The expectation is that this information will be published for all studies that receive an ethical opinion but should you wish to provide a substitute contact point, wish to make a request to defer, or require further information,, please contact the REC Manager Miss Kathryn Murray, nrescommittee.yorkandhumber-sheffield@nhs.net. Under very limited circumstances (e.g. for student research which has received an unfavourable opinion), it may be possible to grant an exemption to the publication of the study.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion of the above research tissue bank on the basis described in the application form and supporting documentation as revised.

Duration of ethical opinion

The favourable opinion is given for a period of five years from the date of this letter and provided

A Research Ethics Committee established by the Health Research Authority