Improving 3D Scaffolds for Skin Tissue Engineering using Advanced Biotechnology

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

Sydney Medical School
The University of Sydney
April 2016
Declaration

I declare that the work described in this thesis is my own except where stated. This work has not been previously submitted for any degree at any institution.

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Conflict of interest disclosure

The author has no affiliation of involvement in any organisation or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.
Acknowledgements

I thank my supervisors’ Dr Zhe Li, Prof Peter Maitz and Dr Yiwei Wang for their support, advice and guidance throughout the study. Their knowledge of and passion for improving the lives of burns survivors has been inspirational. A special thank you to Dr Yiwei Wang who has patiently and wisely guided me through the last four years.

My gratitude goes to Dr Ali Fathi from the School of Chemical and Biomolecular Engineering at The University of Sydney who performed the mechanical investigations in this study and to Prof Qing Li from the School of Aerospace, Mechanical and Mechatronic Engineering for his assistance with analysis of the mechanical investigations. Thank you to Dr Yiwei Wang who supervised the one-step grafting in the animal studies and to Francia Garces-Suarez who assisted with the qPCR analyses.

I am especially grateful to Andrew of the Diagnostic Pathology Unit at Concord Hospital for his invaluable assistance establishing histological protocols and to Dr Mario D’Souza for sharing his statistical knowledge with me. Thank you to Dr Maaike Kockx for her help with the gene expression analysis and to Kate Nieuwendyk and Susan Taggart of the Concord Hospital Burns Unit for their warmth and assistance.

Also, thank you to the staff and students of the ANZAC Research Institute and the Molecular Physiology Unit for their unique and enjoyable camaraderie.

Special thanks to the Ghosh Family Foundation for their unwavering support and generosity, to the Sydney Burns Foundation and National Health and Medical...
Research Council for financial support and to Elastagen for providing the elastin-modified version of Integra Dermal Regeneration Template.

Finally, a very special thank you to my family and friends for their boundless love and optimism.
Publications arising from this work

Publications


Conference proceedings


C. Chong, Y. Wang, A. Fathi, P. Maitz and Z. Li. ‘A Novel Triple Polymer Composite Scaffold for Skin Tissue Engineering’. Poster presentation. TERMIS European Chapter Meeting, Genova, Italy (June 2014).

C. Chong, Y. Wang, C. Blaker, P. Maitz and Z. Li. ‘Collagen-Polycaprolactone biocomposite for skin regeneration’. Oral presentation. 36th Australian and New Zealand Burns Association Annual Scientific Meeting, Hobart, Australia (October 2012).
Y. Wang, C. Chong, C. Blaker, P. Maitz and Z. Li. ‘Modified collagen-PCL scaffolds enhance skin cell ingrowth and promote skin regeneration during wound healing’. Poster presentation. 3rd TERMIS World Congress, Vienna, Austria (September 2012).

C. Chong, Y. Wang, C. Blaker, P. Maitz and Z. Li. ‘The Interactions of Skin Cells with Collagen-Polycaprolactone Scaffolds’. Oral presentation. The Australian Society for Medical Research, Sydney, Australia (June 2012).

Y. Wang, C Chong, C. Blaker, P. Maitz and Z. Li. ‘Skin tissue engineering using a biodegradable polymer’. Poster presentation. 9th Biomaterials World Congress, Chengdu, Chine (June 2012).
Awards, prizes and scholarships

National Health and Medical Research Council Postgraduate Research Scholarship 2012-2016

Concord Repatriation General Hospital Research Travelling Scholarship 2014

Postgraduate Research Support Scheme 2014

Short-listed Finalist, Concord Hospital Early Career Research Prize 2012

Oral Research Presentation Prize, 36th Australian and New Zealand Burns Association Annual Scientific Meeting 2012

Postgraduate Research Support Scheme 2012
Abstract

Existing, dermal, regenerative scaffolds facilitate dermal repair and wound healing of severe burn injuries; however, new tissue is often functionally, mechanically and aesthetically abnormal due to irregular deposition of new extracellular matrix. In the present study two novel, elastin-containing scaffolds were developed, characterised and examined both in vitro and in vivo aiming to minimise wound contraction, improve scar appearance and increase skin elasticity post-healing.

The first types of scaffolds were electrospun from a triple polymer solution of collagen, elastin and poly(ε-caprolactone) (CEP). Two scaffolds were chosen for characterisation: CEP 1 was fabricated using a 1.5 % (w/v) collagen, 12 % (w/v) elastin and 1.5 % (w/v) poly(ε-caprolactone) (PCL) solution, a flow rate of 3 mL/h, an air gap of 15 cm and an applied electric potential of 25 kV; and CEP 2 was electrospun using a 2 % (w/v) collagen, 12 % (w/v) elastin and 1 % (w/v) PCL solution at 1 mL/h, 20 cm and 20 kV. In vitro cell studies using human, dermal fibroblasts (HDFs) and immortalised, human keratinocytes (HaCaTs) revealed CEP 1 and CEP 2 supported cell-seeding and cell proliferation with significantly higher proliferation of both cell types on CEP 1. Additionally, subcutaneous implant studies in mice revealed minimal inflammation in response to both scaffolds with CEP 1 vascularised by week 2 post-surgery. However, CEP 1 was rapidly biodegraded after 2 weeks. Collagen deposition was observed in encapsulating tissue and new tissue with consistent collagen expression over 24 weeks.

The second type of scaffold investigated was an elastin-modified version of the commercial, dermal substitute Integra Dermal Regeneration Template (IDRT). Elastin-IDRT (EDRT) was developed by inclusion of 10% human tropoelastin and then
investigated in comparison with IDRT. Morphological analysis by scanning electron microscope and mechanical characterisation revealed EDRT had significantly enlarged pores, higher porosity and increased deformability. Higher cell seeding efficiency of HaCaTs on EDRT was observed compared to IDRT but cell proliferation rate was found to be similar over 28 days. HDFs displayed increased cell growth rate on EDRT over 28 days compared to IDRT. Enhanced and accelerated HDF infiltration of EDRT was also visualised with complete infiltration by day 14 post-seeding. An *in vivo*, mouse, subcutaneous implant model showed that EDRT induced minimal inflammation. Gene expression of mouse collagen was consistent over 24 weeks with non-significant increases in elastin expression from weeks 2 and 4. One-step grafting demonstrated similar contraction between EDRT-, IDRT- and autografted wounds with final contraction around 40 % compared to 100 % in open wounds. EDRT displayed significantly accelerated, early-stage angiogenesis with higher vascularisation than IDRT-grafted, autografted or open wounds 2 weeks post grafting. By week 4 EDRT- and IDRT-grafted wounds had similar levels of vascularisation which were higher than autografted and open wounds. EDRT showed improved mechanical performance, supported enhanced cell interactions *in vitro* and accelerated angiogenesis *in vivo*.

In summary, investigated scaffolds demonstrated properties that could potentially improve burn wound healing. The inclusion of elastin in scaffolds produced by either electrospinning or lyophilisation improved HDF infiltration and supported formation of a confluent layer of HaCaTs which could result in increased pliability of new skin and accelerated wound healing. In EDRT elastin improved scaffold porosity, pore size and accelerated angiogenesis *in vivo* indicating EDRT can facilitate and improve wound remodelling. Further investigation of both scaffolds is warranted especially due to the
vascular inductive effects of EDRT and the synchronous spatial and temporal biodegradation of CEP 2 observed \textit{in vivo}. 
**Abbreviations**

1.5C12E1.5P  1.5 % (w/v) collagen, 12 % (w/v) elastin, 1.5 % (w/v) PCL

2C12E1P  2 % (w/v) collagen, 12 % (w/v) elastin, 1 % (w/v) PCL

2.5C12E0.5P  2.5 % (w/v) collagen, 12 % (w/v) elastin, 0.5 % (w/v) PCL

3C12E  3 % (w/v) collagen, 12 % (w/v) elastin

7.5C7.5P  7.5 % (w/v) collagen, 7.5 % (w/v) PCL

3D  three dimensional

bp  base pair

cDNA  complementary DNA

CE  collagen-elastin

CEA  cultured epidermal autograft

CEP  collagen-elastin-PCL

CP  collagen-PCL

DAB  diaminobenzidine chromogenic substrate

DMEM  Dulbecco’s Modified Eagle’s Medium

DMSO  dimethyl sulfoxide

dsDNA  double-stranded DNA

ECM  extracellular matrix

EDRT  elastin-modified Integra Dermal Regeneration Template
FBS  fetal bovine serum
FITC  fluorescein isothiocyanate
GAG  glycosaminoglycan
H&E  haematoxylin and eosin
HaCaT  immortalised, human, keratinocyte cell line
HCl  hydrochloric acid
HDF  human, dermal fibroblast
HFP  1,1,1,3,3,3-hexafluoro-2-propanol
HMDS  hexamethyldisilazane
IDRT  Integra Dermal Regeneration Template
IHC  immunohistochemistry
MMP  matrix metalloproteinase
MTS  3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
mRNA  messenger RNA
NHEK  normal, human, epidermal keratinocytes
OCT  optimal cutting temperature
PCR  polymerase chain reaction
PBS  phosphate buffered saline
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<tr>
<td>PCL</td>
<td>poly(ε-caprolactone)</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEO</td>
<td>poly(ethylene oxide)</td>
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<td>PGA</td>
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<td>Ppia</td>
<td>Peptidylprolyl isomerase A</td>
</tr>
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<td>qPCR</td>
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</tr>
<tr>
<td>TBSA</td>
<td>total body surface area</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TIPS</td>
<td>thermally induced phase separation</td>
</tr>
<tr>
<td>UV</td>
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CHAPTER 5 ANIMAL STUDIES OF NOVEL, SKIN SUBSTITUTES ............... 163
Chapter 1

General Introduction
1.1 Human skin structure and function

Skin is the largest organ of the human body housing appendages such as hair, capillaries and sweat glands which provide sensory, thermoregulatory and protective functions (1) (Figure 1.1A). The skin is composed of two main layers, the outermost layer is the epidermis which is interlocked to the inner layer, the dermis (Figure 1.1B). The dermis is also connected to the subcutaneous tissue (hypodermis) which is mainly composed of adipose tissue (1) (Figure 1.1B).

1.1.1 Epidermis

The epidermis is the thinnest skin layer protecting the body from friction and microorganisms (1). There are up to five layers mainly comprised of keratinocytes with other cell types also present, namely, melanocytes which produce melanin, Merkel cells which detect the sensation of touch, Langerhans cells which participate in immune responses and Odland’s bodies which produce a lipid to tightly bind keratinocytes together (1, 2).

Keratinocytes originate from the deepest layer, called the stratum basale (1) (Figure 1.2). Until recently the stratum basale was thought to consist of a pool of stem cells called transit-amplifying cells located in specific locations which underwent several rounds of division to amplify the keratinocyte population (3, 4). These keratinocytes would be differentiated and keratinised as they migrated upwards (5). Keratinisation is the deposition of keratin, a tough, pliable protein, in keratinocytes (6).
However, recent findings in xenografted human foreskin have shown that where the human epidermis has an undulating basal layer and varying number of strata, epidermal proliferation units are organised into narrow compartments with cross-sections ranging from one to ten and cells would migrate perpendicular to the skin surface. There was no preferred site of origin of the epidermal proliferation units with sites located on the relatively flat regions of the basement membrane, the side of the rete ridges, the base of the rete ridges and the tip of the dermal papillae with no specialised structure or niche for stem cells (7).
Figure 1.1: (A) Schematic representation of skin layers and appendages, adapted from (8); and (B) image of haematoxylin and eosin (H&E) stained skin (9).
Figure 1.2: Image of H&E stained human skin showing epidermal layers in thin skin (A) and thick skin (B). Adapted from (9).

1.1.2 Basement membrane

The basement membrane acts as a physical barrier between the epidermis and dermis while also serving to connect the two layers with proteins such as anchoring fibril type VII collagen (10). It is a complex assembly of proteins such as type IV collagen, laminin and heparin sulphate proteoglycans (11). It also regulates the movement of cells and molecules between the two layers binding a range of cytokines and growth factors to control release during wound remodelling and repair (12, 13). Research also suggests the basement membrane affects the regenerative ability of skin by keeping stem cells in their niche through very strong interaction and adherence between the two (14, 15).
1.1.3 Dermis

The dermis houses most of the skin appendages and is responsible for providing nutrients and physical support to the epidermis via rete ridges (16, 17). The dermis encompasses vascular networks which provide nutrient and waste exchange for cells, nerve networks permitting interaction with the environment and keratinised appendage structures such as nails which provide protection (18, 19). The dermis is structurally subdivided into two layers; the upper, papillary layer and lower, reticular layer (2) (Figure 1.3A). The papillary layer of the dermis has a network of fine collagen and elastin fibres which compose the loosely connected tissue which surround a large amount of nerves, capillaries, water and cells (2). The finger-like projections, called dermal papillae, originate from this layer and connect the dermis to the epidermis forming unique fingerprints in humans (20). The reticular dermis is more dense and irregular than the papillary layer with thick bundles of interlacing collagen and elastin fibres which are mostly orientated parallel to the skin exterior (20). There are also fewer nerves and capillaries compared to the papillary dermis (2) (Figure 1.3B). This combination of collagen and elastin fibres in the reticular layer gives skin its strength and elasticity (21).

The dermis is much thicker than the epidermis and mostly consists of collagen and elastin fibres (1, 2). Collagen accounts for approximately 70 % of the dry weight of dermis and is responsible for the tensile strength of skin preventing skin from tearing when stretched. There are several types of collagen present in the dermis including types I, III, V, VI, XII, XIV and XVI (22, 23). Elastin accounts for 2-4 % of the dry weight of skin (24) and has thinner fibres than collagen. These fibres are interwoven among the collagen bundles returning skin to its normal position after stretching (1). Collagen and elastin are synthesised by fibroblasts, the main cell in the dermis.
Figure 1.3: (A) Image of H&E stained skin showing dermal layers in thin skin and (B) image of van Gieson stained skin showing collagen (pink) and elastin (black) fibres in thick skin. Adapted from (9).

1.2 Burn injuries

Damage to skin can be incurred numerous ways ranging from surgically removing skin defects, such as nevi, to involvement in a vehicular accident or fire. At times, surgical intervention is required when tissue has been extensively damaged and is beyond its ability to repair itself. Specifically focussing on burn injuries, such damage or destruction of skin can be inflicted by heat, cold, electricity, chemicals, radiation or friction. Severe burns are one of the most debilitating injuries requiring prolonged hospital stays and often resulting in not only physical scarring but emotional trauma. Globally, there were nearly 11 million incidences of burns requiring medical attention (25).
Burn injuries are classified according to the depth of the burn (Figure 1.4) and, in conjunction with the affected total body surface area (TBSA), treated accordingly. Burn depth is related to the amount of epidermis and dermis damaged and this determines the skin’s ability to achieve wound healing autonomously or whether surgical treatment of the wound is required (26, 27).

![Figure 1.4: Schematic of burn depth classification demonstrating the layers of skin damaged. Adapted from (28).](image)

According to Evers et al. (2010) (29) a superficial burn can be caused by excessive sun exposure or by short exposure to hot liquids and only affects the epidermis. The burn does not blister but is moist with a pink to red appearance and can be moderately to severely painful. The burn heals spontaneously within a week usually with no scarring due to regenerative, epidermal, stem cells present at the base of hair follicles in the undamaged dermis.

Partial-thickness burns can be further subdivided into superficial dermal and deep dermal burns. Superficial dermal burns are caused by hot liquids, weak acids and also flash burns (29). This type of burn affects the epidermis and papillary dermis resulting in blistering and redness but nerves are intact so the pain is severe. Superficial, dermal
burns can regenerate from the viable stratum basale of surrounding undamaged tissue in conjunction with stem cells from surviving hair follicles in the wound area (18, 26, 30). Wound healing can take up to 3 weeks with pigment changes possibly occurring. Deep dermal burns could be caused by flame, chemical, electrical or hot liquid burns (29). The epidermis, papillary dermis and reticular dermis are affected with injured skin appearing dry and white. There is minimal pain due to loss of epidermal appendages. Deep, dermal burns heal through formation of granulation tissue over 3-6 weeks. Fibroblasts in this tissue produce collagen and differentiate into contractile myofibroblasts which rapidly close the wound along with migration of basal keratinocytes from edges of the wound (26, 30). This form of healing results in varying degrees of scarring and contraction depending on the extent of damage to the dermis.

Full-thickness burns are the most severe form and they affect the full thickness of skin extending to the subcutaneous tissue or deeper. These burns are caused by flame, electrical, chemical, blast or self-immolation and damaged tissue appears leathery, dry and white or red. There is no pain associated with deep burns due to the complete destruction of nerves and other appendages in the affected area. Treatment of such injuries requires removal of necrotic tissue and assisted wound closure with the use of skin grafts or substitutes to minimise infection and sepsis; however, there is usually significant scarring and contraction.

1.3 Wound healing

Wound healing is a rapid process which has evolved to allow humans to recover tissue integrity using scarring to join the edges of wounds or to fill voids of tissue resulting from dermal loss (31). This repair process is sequential with four overlapping stages: haemostasis, inflammation, proliferation and remodelling and is an event-driven
cascade triggered by one cell type propelling the wound through the phases of wound healing (32).

Adult wound healing begins immediately with haemostasis in which blood flow to the injured area is limited by vasoconstriction and platelets released from the damaged blood vessels adhere to collagen at the edge of damaged vessels forming a plug and activating the clotting cascade (33, 34). The platelet plug is secured by a fibrin clot at the site of injury which holds the damaged tissue together and provides a provisional matrix to support cell migration (34, 35). Platelets also release cytokines and growth factors which recruit neutrophils and macrophages advancing the process of wound healing to the inflammatory stage (36, 37).

The inflammatory response begins with vasodilation of blood vessels and increasing vascular permeability to allow fluids to pass through the vessel walls (38) giving the classical signs of inflammation with the area surrounding the wound appearing red, hot and swollen (39, 40). The neutrophils attracted to the area squeeze through the porous walls and migrate to the wound area (41) reaching a maximum concentration 24-48 hours after injury and declining thereafter. The neutrophils phagocytose foreign particles, bacteria and devitalised tissue by secreting proteases and free radicals which also have a bactericidal effect (42). The dead neutrophils are removed from the area by macrophages which are derived from monocytes. This prevents disintegrating neutrophils from continuing to release proteases and cytokines, which would prolong the inflammatory phase thereby allow the wound to progress to the proliferative phase (43).

The proliferative phase is when granulation tissue begins to fill the wound and consists of angiogenesis and the deposition of collagen by fibroblasts to form a new matrix.
Fibroblasts are attracted to the wound by the growth factors released from platelets and macrophages (44) and migrate over the provisional fibrin matrix proliferating and depositing new ECM (34). As fibroblasts have a limited replicative ability they eventually senesce (45) and removal of accumulated senescent fibroblasts helps to stimulate wound healing (46). Concurrently, endothelial cells lining the blood vessels in the wound are activated and begin to divide sprouting or budding through the vessel basement membrane in the new matrix (47). New blood vessels form where the tips of new capillary buds meet (35, 47).

Re-epithelialisation occurs around the same time with basal keratinocytes migrating across the granulation tissue. Stem cells also migrate from within the dermis and keratinocytes migrate from the wound edge to establish a new skin barrier. Once this has been achieved keratinocytes resume a basal cell phenotype differentiating into a stratified, squamous, keratinising epidermis (48). The source of stem cells from within the dermis reside at the base of hair follicle bulges (49) and it is thought that these cells migrate to the surface to aid re-epithelialisation (50) and contribute to the regeneration of sebaceous glands and hair follicles (49). The final phases of the inflammatory response and epithelialisation coincide with the formation of granulation tissue and migration of fibroblasts and endothelial cells (51).

The final phase of wound healing is remodelling of scar tissue which is partly performed by matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs produced by fibroblasts and macrophages and can last several months. In adults, scar formation is characteristic (52) with the balance of newly formed collagen and destruction of old collagen determining the final physical characteristics of the scar (51). There are three types of scars: atrophic scars are depressions in the skin; hypertrophic scars are elevated and can subside with time; and keloid scars are
elevated, expansive and continue to grow beyond the margin of the original wound (51).

Scarring of wounds is thought to be caused by high expressions of two isoforms of the TGF-β family, TGF-β1 and TGF-β2. During wound remodelling, inflammatory cells, such as monocytes and macrophages, and the degranulation of platelets, release TGF-β1 and TGF-β2. These two isoforms are heavily implicated as a cause of scarring in adults. Application of neutralising antibodies to TGF-β1 and/or TGF-β2 markedly reduced scarring in rodent wounds (53-55) with no delays in wound healing (56). Comparatively fetal wounds, which heal by regeneration rather than repair, have very low levels of TGF-β1 and TGF-β2 and very high levels of TGF-β3 (51). Additionally, adult wounds treated exogenously to attain TGF-β3 levels similar to those in scar-free embryonic healing resulted in improved or absent scarring (55).

More extensive injuries in which large areas of tissue are damaged or lost pose even more serious issues. If left untreated these wounds result in severe contraction which occurs when surrounding skin pulls together to more quickly close a wound and this can result in a loss of mobility while also having a detrimental, psychological impact. These wounds may also be unable to achieve autonomous wound closure as they are likely to have no blood supply to initiate clotting and begin the wound healing cascade. The extensive tissue damage or loss would also result in the absence of regenerative elements such as stem cells. In such situations, surgical intervention is required using treatments which can provide wound closure as well as provide guidance for wound healing.
1.4 **Skin grafts and substitutes used for the treatment of burn injuries**

1.4.1 **Skin grafts**

The gold-standard treatment of partial- and full-thickness burn injuries is the early excision of necrotic tissue followed by autografting. Autografts are autologous skin consisting of the epidermis and dermis excised from healthy, undamaged sites of the patient which are grafted onto a clean wound bed (57) (Figure 1.5). They can be full-thickness grafts, consisting of the epidermis and whole dermis, or split-thickness, consisting of epidermis and the upper part of the dermis (51). A disadvantage of full-thickness autografts compared to split-thickness autografts is that full-thickness autografts they are less likely to vascularise and, therefore, likelihood of graft rejection is higher (51). Autografts provide wound coverage while reducing wound contraction by reintroducing regenerative elements.

Unfortunately, a shortage of skin donor sites can occur with burn survivors who have more than 50 % TBSA (58, 59). This can be overcome by repeated harvesting of donor sites over a period; however, healing of harvest sites can be slow with additional scarring and possible pigmentation changes occurring. Therefore, autografts themselves are not an ideal solution as additional injury is inflicted on the patient.
Figure 1.5: Schematic of autograft harvest and application. Adapted from (60).

Two biologically sourced alternatives investigated as substitutes for autografts were allografts and xenografts. Allografting is transplantation between individuals of the same species with skin tissue sourced from cadavers. Xenografting is transplantation between individuals of different species. Both alternatives have proved useful as temporary dressings which improve the quality of the wound for further treatment but they are not permanent replacements as graft rejection occurs due to the immune system rejecting the foreign objects (61). Additionally, allografts and xenografts have limited supply and inconsistent quality with the risk of pathogen transfer between the donor and recipient (27).

1.4.2 Skin substitutes

In response to the need for reliable, skin substitutes the field of skin tissue engineering evolved. It is an interdisciplinary field focussing on the generation of new tissue for replacement, repair or regeneration of damaged skin. Skin tissue engineering aims to create a low-cost, reproducible substitute capable of promoting and accelerating the regeneration of new, skin tissue possessing normal structural and functional properties. Current, manufactured substitutes have been developed which are able to
replace damaged epidermal and/or dermal layers of skin (62) (Table 1.1) and are able to improve wound healing to an extent.

1.4.2.1 **Acellular wound dressings**

Biobrane is a biosynthetic dressing available for treatment of superficial and partial-thickness injuries. It is a temporary skin dressing composed of a silicone membrane bonded to a nylon mesh with porcine, dermal, collagen peptides. Studies found Biobrane remained attached to a range of skin injuries, including donor sites and meshed autografts, until re-epithelialisation occurred (63). Its use was also observed to reduce pain, permit range of motion when applied over joints, usually had no fluid accumulation underneath the dressing and its translucency allowed direct observation of healing process (63-66).

1.4.2.2 **Cultured epidermal substitutes**

One of the greatest developments in skin tissue engineering was the cultivation of human keratinocytes in the late 1970s (67, 68). This achievement permitted the expansion of a patient's own keratinocytes to form cell sheets which could be grafted back onto the patient as an epidermal substitute. These sheets of keratinocytes were termed cultured epithelial autografts (CEAs) and have been shown to aid dermal regeneration with smoother graft appearance and more even pigmentation than autografts (69, 70).
### Table 1.1: Examples of skin substitutes used in the treatment of skin injuries (51, 62, 71-75).

<table>
<thead>
<tr>
<th>Product</th>
<th>Epidermal component</th>
<th>Dermal component</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Epidermal substitutes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cultured epithelial autografts</td>
<td>sheet of autologous keratinocytes grown from skin biopsy</td>
<td>none</td>
<td>cover large area with little rejection risk; aid dermal regeneration; smoother graft appearance</td>
<td>3 weeks required to produce fragile sheets; susceptible to blistering</td>
</tr>
<tr>
<td>Epicel (Genzyme Tissue repair Corp.)</td>
<td>cultured epithelial autografts grown on petrolatum gauze backing</td>
<td>none</td>
<td>large area of permanent wound coverage with little risk of rejection</td>
<td>3 weeks required to produce fragile, confluent sheets; susceptible to blistering</td>
</tr>
<tr>
<td><strong>Dermal substitutes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alloderm (Life Cell Corporation)</td>
<td>processed cadaver allograft skin</td>
<td>none</td>
<td>processing reduces antigenic components; successful resurfacing of full-thickness burns</td>
<td>graft rejection and risk of disease transfer</td>
</tr>
<tr>
<td>Permacol (Tissue Sciences Laboratories)</td>
<td>porcine-derived acellular dermal matrix</td>
<td>Bovine collagens type I, II and V and bovine α-elastin from ligamentum nuchae</td>
<td>non-immunogenic due to removal of non-collagenous and cellular materials; supports fibroblast infiltration and revascularisation</td>
<td>revascularisation sometimes inefficient to support epidermal graft</td>
</tr>
<tr>
<td>MatriDerm (Skin and Health Care AG)</td>
<td>silicone layer</td>
<td>bovine, collagen type I and chondroitin-6-sulphate</td>
<td>improves skin pliability; reduces contraction; stimulates dermal regeneration</td>
<td>mixed results with pliability; risk of disease transfer</td>
</tr>
<tr>
<td>Integra (Integra Life Sciences Corp.)</td>
<td>thin silicone layer (Biobrane)</td>
<td>collagen-coated nylon mesh seeded with neonatal, allogeneic fibroblasts</td>
<td>promotes fibroblasts and epithelial cell ingrowth; silicone replaced with an autograft after vascularisation</td>
<td>bovine collagen is antigenic and a disease risk; 3 weeks required to expand dermal autograft</td>
</tr>
<tr>
<td>TransCyte (Advance BioHealing, Inc.)</td>
<td>dermal fibroblasts secrete collagen, glycosaminoglycan and growth factors to aid wound healing</td>
<td>nylon mesh not biodegradable; rejection and disease risk from fibroblasts</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Bilayered substitutes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autografts</td>
<td>epidermis harvested from healthy site of patient</td>
<td>dermis harvested from healthy site of patient</td>
<td>gold-standard treatment; replaces regenerative elements; good cosmetic results</td>
<td>revascularisation sometimes inefficient leading to graft rejection; additional scarring</td>
</tr>
<tr>
<td>Apligraf (Organogenesis)</td>
<td>allogeneic, neonatal keratinocytes</td>
<td>neonatal, allogeneic, foreskin fibroblasts on bovine, collagen type I</td>
<td>comparable graft take to autografts; good cosmetic results; no signs of rejection observed</td>
<td>risk of chronic graft rejection and disease from allogeneic cells; requires repeated applications</td>
</tr>
<tr>
<td>OrCel (Ortec International)</td>
<td>allogeneic, neonatal keratinocytes</td>
<td>neonatal, allogeneic, foreskin fibroblasts in bovine, collagen sponge</td>
<td>favourable environment for host cell migration and provides source of cytokines and growth factors</td>
<td>temporary skin replacement; designed as dressing; risk of rejection and disease</td>
</tr>
</tbody>
</table>
CEAs remain a common choice for the treatment of burn injuries to this day; however, they have numerous disadvantages including lengthy hospital stays with 2-3 weeks required for cultivation of cells (58). CEAs are also difficult to manipulate due to their fragility and inherently thin nature (58). Furthermore, the enzymatic treatment used to detach the CEAs from culture dishes is thought to lead to poor basement membrane formation (62) which in turn is responsible for blistering and variable graft take ranging from 0 % to more than 80 % (76, 77). CEAs can also take several years to develop normal histological appearance (78). CEAs were originally intended to replace autografts in the treatment of deep burns; however, use of CEAs alone have been shown to be unsuitable as they do not replace the damaged dermis and its components which are necessary for normal skin function and appearance (67, 79).

1.4.2.3 **Acellular dermal substitutes**

Skin substitutes designed for dermal replacement aim to guide regeneration of skin tissue as the complexity of ECM makes it difficult to replicate. There are several dermal substitutes commercially available, the most popular of those is Integra Dermal Regeneration Template (IDRT; Integra Life Sciences Corporation, Plainsboro, NJ, USA), a collagen-based matrix developed in the early 1980s (80-84). It is a porous, acellular substitute fabricated by lyophilising a solution of bovine, collagen type I and chondroitin-6-sulphate to form the dermal layer. The temporary, epidermal layer consists of silicone and acts as a barrier to prevent fluid and heat loss as well as minimising bacterial infection (Figure 1.6). The collagen matrix serves as a biodegradable support and provides guidance for cell infiltration of the wound area. After the collagen matrix is vascularised the silicone layer is replaced by a thin, split-thickness autograft to form the new, epidermal layer. Integra is also commercially
available as a single layer, silicone free substitute intended for the treatment of deep dermal skin injuries.

Figure 1.6: Schematic of an IDRT graft. Adapted from (85).

IDRT is used in the treatment of deep-partial to full-thickness burn injuries when a shortage of skin donor sites are available (86). The use of IDRT has been seen to improve epidermal graft take while also decreasing hypertrophic scarring and improving the cosmetic outcome of new skin (87-89). However, although IDRT is a commonly used substitute there are high costs involved and multiple surgeries required with high risks of infection, limited graft take, wound contraction and scarring (90, 91).

MatriDerm (Skin and Health Care AG, Billerbeck, Germany) is another commercial substitute and it is the only skin substitute available containing elastin, the critical matrix protein in the elastic network of human dermis. MatriDerm is fabricated by lyophilisation of bovine collagen to form a non-cross-linked matrix which is then coated with 3 % α-elastin derived from bovine ligament nuchae. Studies of MatriDerm have returned mixed results with one study showing improved results of hand wounds.
treated with MatriDerm and non-meshed skin grafts compared to conventionally grafted wounds (92-94). Other benefits also included reduced wound contraction and stimulation of dermal regeneration (95). However, a long-term clinical trial showed no difference in scar elasticity between MatriDerm and split-thickness grafts alone (96).

1.4.2.4  **Cultured, dermal substitutes**

Further development of dermal substitutes have investigated cellular substitutes in which wound healing is stimulated by the seeding of fibroblasts into the scaffold to promote ECM synthesis (97). One such study demonstrated that a gelatin-based scaffold in a mouse model enhanced re-epithelialisation of full-thickness wounds grafted with fibroblast-seeded scaffolds compared to wounds grafted with acellular scaffolds (98). Another study with pigs showed that improved tissue regeneration, decreased wound contraction and less scarring was correlated with increasing fibroblast numbers (99). Clearly, the inclusion of fibroblasts has promising results in the improvement of wound healing.

Cellular dermal substitutes are commercially available, for example TransCyte (Advanced BioHealing, Inc., La Jolla, CA, USA) contains allogeneic, neonatal fibroblasts cultured for 17 days *in vitro* on nylon fibres. The fibres are embedded in a silastic layer allowing cells to synthesise ECM and growth factors before being rendered nonviable by freezing (97, 100, 101). Accelerated wound healing and decreased inflammation has been observed with the use of TransCyte when compared to the use of split-thickness grafts in partial-thickness burns (102, 103).
1.4.2.5 **Cultured, bilayered skin substitutes**

Bilayered, cellular skin substitutes, also known as living skin substitutes, have been developed and have great potential to improve and simplify treatment with both the epidermal and dermal components being replaced in a one-step procedure (79, 97). Currently, the components used to construct bilayered constructs are simple. For example, Apligraf (Organogenesis, Canton, MA, USA) is composed of allogeneic, neonatal fibroblasts and keratinocytes cultured on a type I, bovine, collagen scaffold (104). Apligraf has extensive studies with treatment of venous and diabetic ulcers (105) and a study examining its use in burn wound treatment demonstrated that a combination of Apligraf with meshed, split-thickness grafts improved skin pigmentation, pliability and vascularisation compared to meshed grafts alone (106).

1.4.2.6 **Issues with existing, commercial skin substitutes**

Current, skin substitutes provide alternative treatment options which can improve clinical outcomes. Epidermal replacements provide wound closure and promote wound healing while dermal replacements can reduce wound contraction and replace lost elements required for regeneration such as basal keratinocytes and stem cells located at the base of hair follicles. The dermal substitutes provide guidance for cell infiltration and wound remodelling and the new skin generated has a more natural appearance with reduced scarring than if a wound is left untreated. However, scar tissue is often functionally, mechanically and aesthetically abnormal as the internal architecture provided by substitutes does not adequately replicate the complex internal structure of normal skin tissue required to direct tissue growth (107).

For example, many substitutes suffer from poor graft take resulting from insufficient, early-stage vascularisation. A blood supply is vital to maintain long-term survival and
integration of the graft, otherwise cells in the substitute die and the construct will slough away. A recent study demonstrated central blood vessels of an autograft were refilled between 48-60 hrs after grafting and this was attributed to early anastomoses forming between the graft and bed vessels in the surrounding undamaged skin (108). This indicates the incorporation of prefabricated vessels into a substitute or the use of materials with biosignals which accelerate angiogenesis could improve treatment by preventing delays in vascularisation and thereby decrease the chances of graft rejection.

Absence of differentiated structures and the physical appearance in scar tissue are another two serious issues encountered with current substitutes. The absence of sweat glands results in skin unable to perform homeostatic functions such as thermoregulation, missing melanocytes leads to a lack of melanin deposition in keratinocytes and thereby increased ultraviolet (UV) sensitivity and variations in skin colour and the lack of Langerhans cells leads to compromised immune defence in skin. Current substitutes do not provide the necessary signals to trigger regeneration of all the components of skin. However, one study has shown the ability of a mixture of melanocytes, fibroblasts and keratinocytes to spontaneously sort on a skin substitute (109). This highlights the ability of skin substitutes to function as a delivery device for cell mixtures while providing a three-dimensional (3D) environment conducive to normal cell behaviour. The inclusion of chemotactic materials or signals in the scaffold could further assist regeneration.

The lack of elasticity in scar tissue is yet another issue arising from the use of current substitutes. Inelastic skin restricts movement especially if the affected area is one such as knees, fingers or shoulders. It has been seen that some substitutes, such as MatriDerm, can sometimes regenerate skin with normal elasticity and flexibility.
However, variable and unreliable outcomes such as these are highly undesirable and further development of scaffolds is necessary. Attention should focus on improving the internal structure of substitutes and the effect on the deposition of new ECM which is central in defining the properties of new tissue. For example, the elastic network of normal, skin tissue relies heavily on the formation of mature, elastin fibres to provide strength and elasticity (110). Internal architecture and composition which closely mimics that of normal ECM would likely be beneficial to regeneration of normal tissue.

1.5 Ultimate goal of skin tissue engineering

Skin tissue engineering is a multi- and interdisciplinary field encompassing fields such as polymer chemistry, biochemistry and stem cell research. Skin tissue engineering aims to combine the knowledge of these fields to development a substitute which can be rapidly produced and regenerate skin to its normal mechanical, functional and aesthetic properties. This includes regeneration of: the ECM to provide support and guidance; the vascular network to improve graft take; skin appendages for functions such as thermoregulation and sensitivity; and the different cell types required for protection.

There are essentially two approaches to this goal. The first is the development of a highly sophisticated, biodegradable scaffold capable of releasing a cocktail of signalling molecules in a temporally and spatially controlled manner to facilitate cell migration, adhesion and essentially skin regeneration. The second approach is to engineer a minimally designed, temporary scaffold which would act as a carrier for undifferentiated or stem cells that would interact to regenerate the skin (51). Both approaches require the creation of a 3D environment which can support cell interactions and promote wound regeneration. It is with this necessity at the forefront
of skin tissue engineering that there are a burgeoning number of investigations into a wide array of scaffold materials and techniques.

1.6 **Materials investigated for skin tissue engineering**

1.6.1 **Natural materials**

Natural materials are biologically-derived components such as collagen, elastin, silk, chitosan and fibronectin. There is focus on the use of natural polymers to fabricate skin substitutes as they provide the advantages of constructing biocompatible and biodegradable scaffolds with non-toxic degradation products. Natural polymers are attractive due to their evolutionarily-developed peptides which provide cell signals to promote wound healing. The disadvantages of natural polymers are batch-to-batch variation, risk of immune rejection and risk of pathogen transfer.

1.6.1.1 **Silks**

Silks have only recently begun to be researched as natural biomaterials for tissue engineering despite their clinical use as sutures for centuries. They are lightweight polymers with tensile strength similar to Kevlar 49 while being more elastic and requiring more energy to break than Kevlar 49 (111, 112). Silks are also thermally stable up to around 250 °C allowing a wide range of processing temperatures (113). The most commonly studied silks are cocoon silk from the silkworm *Bombyx mori* and dragline silk from the spider *Nephila clavipes* (114-118).

Silks are composed of a filament core protein, called silk fibroin, with a glue-like coating consisting of a family of sericin proteins (111). Silks from *B. mori* and *N. clavipes* are
structurally characterised by block copolymers of hydrophobic blocks with highly preserved repetitive sequences of short side-chain amino acids and hydrophilic blocks composed of more complex sequences of larger side-chain and charged amino acids (119, 120). The hydrophobic blocks give rise to the tensile strength of silk fibroin by forming β-sheets or crystals via hydrophobic interactions or hydrogen bonding (121, 122) and the elasticity and toughness of silk arises from the combination of these hydrophobic blocks with less ordered hydrophilic blocks (114, 117, 123). The repetitive hydrophobic regions are the basis for genetic engineering of host systems, such as *Escherichia coli*, yeast, mammalian cells and plants, to produce recombinant silk fibroin-like proteins which exhibit low water solubility due to hydrophobicity (118, 121, 122, 124-133).

Many studies have shown the ability of silkworm silk to support human fibroblast attachment and proliferation (134-137); however, it has been identified that sericin is a major cause of adverse immune responses (138). Removal of sericin and regeneration of silk fibroin has shown good biocompatibility (138-141), haemocompatibility (142) and oxygen and water permeability (143, 144). It has also been reported that the use of silk fibroin films and composites in wound dressings promote *in vivo* healing (145, 146).

1.6.1.2 *Chitosan*

Chitosan is produced through the partial deacetylation of chitin, which is the second most abundant natural polymer obtained from the exoskeletons of crustaceans (147). It is a linear polysaccharide composed of glucosamine and *N*-acetyl glucosamine units linked by β (1-4) glycosidic bonds (148). The hydroxyl and amino groups can be modified to synthesise various derivatives of chitosan (149-151). The content of
glucosamine is called the degree of deacetylation which varies from 30-95% depending on the source and preparation procedure and this also determines the molecular weight (152, 153).

Chitosan is insoluble in aqueous solutions above pH 7; however, the protonated free amino groups on glucosamine facilitate chitosan’s solubility in dilute acids (pH <6.0) (154). Conversely, the ionic groups make techniques such as electrospinning challenging as the cationic groups require a solvent that forms a salt with chitosan to destroy the interactions between neighbouring chitosan molecules (155-158). Chitosan’s cationic groups also facilitate pH-dependent electrostatic interactions with anionic glycosaminoglycans (GAG) and proteoglycans (159).

Among the benefits of chitosan, its biocompatibility, biodegradability and bioactivity have made it a polymer of interest in skin tissue engineering (160, 161). Also of interest are chitosan’s properties to achieve haemostasis, accelerate tissue regeneration and stimulate collagen synthesis by fibroblasts (162-165). Chitosan’s properties have also been seen to persist or improve when used to make double-polymer scaffolds. For example, a chitosan-alginate polyelectrolyte complex membrane showed greater stability to pH changes than either material alone which provides more effect controlled-release membranes (166). This ability to retain properties after blending is useful in skin tissue engineering as it allows the combination of multiple materials to produce a scaffold with enhanced capabilities.

1.6.1.3 Collagen type I

‘Collagen’ is a generic term for proteins forming a characteristic triple helix of three polypeptide chains and, based on structure and supramolecular organisation, can be grouped into fibril-forming, fibril-associated, network-forming, anchoring fibril,
transmembrane, basement membrane or other collagens with unique functions (167). Collagens also have functional properties providing cell signals for attachment and proliferation (168). All collagens have a characteristic supramolecular structure that is a right-handed triple helix (169, 170) and each of the α-chains form an extended left-handed helix with a pitch of 18 amino acids per turn (171). Collagen can be homotrimers consisting of three identical chains or heterotrimeric consisting of two or three different chains. The chains are staggered by one residue relative to each other and supercoiled around a central axis (172). Close packing of the α-chains is permitted around this axis by use of glycine at every third residue which is positioned in the centre of the triple helix while the more bulky side chains of other amino acids are located in the outer positions (167, 173).

Networks of fibril-forming collagens provide a dynamic, 3D environment in tissues such as skin, bone and articular cartilage and include collagen types I, II, IV, V and XI (167). They are characterised by their ability to assemble into highly orientated supramolecular aggregates and, under SEM, have a banding pattern with periodicity of about 70 nm (174). Collagen type I is the most abundant and well-studied collagen providing tensile stiffness in fascia (Figure 1.7). It is a heterotrimeric collagen with two \( \alpha_1 \)-chains encoded in the 17q21.31-q22 region in humans and one \( \alpha_2 \)-chain encoded in the 7q22.1 region (167). A previous study has demonstrated the ability collagen type I to support the formation of neo-follicles when a collagen-GAG scaffold was cultured with murine dermal papilla cells (175) indicating collagen provided the required structure and signals for development of the neo-follicles.
1.6.1.4 **Elastin**

Elastin is another natural polymer steadily garnering interest in skin tissue engineering. The monomer form of elastin is called tropoelastin which undergoes cross-linking to form the insoluble biopolymer, elastin (176). Tropoelastin is encoded by a single copy gene in humans located in the 7q12.2 region (177) and is characterised by alternating hydrophobic and hydrophilic domains (178). These alternating domains are involved in different functions of the protein with the hydrophobic domains implicated in monomer association and elastic function while hydrophilic domains are involved in polymerisation of the monomer through cross-linking (179).

Elastin is found in elastic tissues such as large arteries, lung, tendons and skin (180) and is produced by several cell types including fibroblast, smooth muscle cells and endothelial cells (181). Elastogenesis primarily occurs during late fetal and early neonatal periods with little elastin turnover occurring in healthy adult tissue (182). Elastin is extremely durable with a half-life of approximately 70 years (183). Elastin is one of two distinct components responsible for the composition of elastic fibres, elastin is the core around which is wrapped a sheath of microfibrils 10-12 nm wide (184).
Elastin is well-known for imparting elasticity, flexibility and resilience to skin while also determining skin texture and quality (183). In damaged skin, elastin has been shown to be reduced or even absent which accounts for the reduced pliability of scar tissue (185).

Elastin also has inherent cell signalling properties including chemotaxis, cell attachment, proliferation and differentiation (178, 186). These structural and functional properties make elastin an attractive polymer for use in skin tissue engineering with the expectation that inclusion of elastin in a skin substitute could increase a substitute’s elasticity while possibly improving cell-scaffold interactions. Interestingly, the presence of enzymatic elastin products have been seen to have stimulatory effects on elastin and collagen production (187). Dermal fibroblasts cultured with elastin products had significantly higher production of elastin and collagen fibres. This increase in elastic fibre deposition was also seen in cultures of skin explants and, furthermore, increased elastic fibre production by human, dermal fibroblasts (HDFs) was translated into athymic nude mice upon injection of HDFs (187).

1.6.2 Synthetic polymers

Synthetic polymers are manufactured polymers which are readily available. Biodegradable, biocompatible and/or bioresorbable synthetic polymers are of interest in skin tissue engineering as the polymer can be degraded and removed via the body’s natural pathways with no surgical intervention. The batch-to-batch consistency of synthetic polymers is also advantageous as mechanical properties, such as tensile strength, are predictable allowing for reliable treatment outcomes. However, synthetic polymers also lack the biological signals inherent in natural polymers. There are many synthetic polymers under investigation for use in skin tissue engineering such as
poly(lactic acid) (PLA) (188), poly(glycolic acid) (PGA) (189) and polycaprolactone (PCL) (190). In this study the only synthetic polymer used was PCL, which is discussed below.

1.6.2.1 **Polycaprolactone (PCL)**

PCL is an aliphatic polyester biodegraded by hydrolysis and has been approved by the Food and Drug Administration (191, 192) (Figure 1.9). It was first synthesised in the 1930s and has regained attention in the tissue engineering field due to its biocompatibility, high tensile strength and controllable biodegradability (193, 194). Degradation of PCL can span months to years depending on polymer properties such as molecular weight, degree of crystallinity and degradation conditions (195-197). The degradation products of PCL are also non-toxic unlike other synthetic polymers such as PLA which can cause mild inflammation (173, 198).

![Figure 1.8: Chemical structure of PCL.](image)

There have been clinical trials of supercutaneously implanted PCL capsules that indicate PCL was well-tolerated over 40 weeks with other studies using PCL as a release vehicle for drugs (199-201). One study developed an ultrathin PCL film as a wound dressing and when tested in rat and pig models the PCL films had a lower level of fibrosis compared to control, non-dressed wounds. There was also no inflammation induced by PCL films with the wound dressing supporting normal wound healing in both partial- and full-thickness wounds (190).
1.6.2.2 *Poly(D,L-lactic-co-glycolic acid) (PLGA)*

PLGA is a family of FDA-approved biodegradable, biocompatible polymers and is popular due to its long clinical experience and possibilities for sustained drug delivery despite the degradation products being mildly inflammatory. It is a copolymer of PLA and PGA and contains equal ratios of the optically active D and L forms of PLA which has an asymmetric α-carbon, PDLA and PLLA respectively (202). Generally, PLA can be highly crystalline in the PLLA form or completely amorphous in its PDLA form whereas PGA is highly crystalline.

PLGA is soluble in a wide range of common solvents such as acetone and ethyl acetate (203, 204). When in water PLGA undergoes degradation by hydrolysis at its ester linkages. Therefore, the rate of degradation can be decreased by including more of the comparatively less hydrophilic PLA to decrease water absorption and subsequently slow down degradation (202). Physical properties of PLGA have also been shown to depend on other factors such as storage temperature and molecular weight which affects mechanical strength (205).

Since PLGA has poor surface chemistry it is widely incorporated with natural polymers to improve cellular interactions and proliferation. One such study has investigated a knitted PLGA mesh with web-like bovine collagen type I sponges in the interfibre spaces of the PLGA mesh (206). The blended scaffolds were shown to support HDF attachment and proliferation with more homogenous cell and ECM distribution than PLGA-only scaffolds. Further *in vivo* experiments of the scaffold as a living dermal substitute in a nude mouse model demonstrated tissue remodelling after 2 weeks and full re-epithelialisation within 4 weeks.
1.6.3 **Composite scaffolds**

Natural and synthetic polymers each have their advantages and disadvantages and one approach to address these shortcomings is to produce scaffolds with a blend of polymers. Composite scaffolds aim to utilise the advantageous properties of both types of polymers during fabrication resulting in a scaffold with improved properties such as increased mechanical strength and enhanced cell signalling abilities. Many different combinations have been investigated with a heavy focus on collagen.

1.7 **Scaffold fabrication methods investigated for tissue engineering**

Scaffold structure is greatly influenced by the scaffold fabrication method employed and there are a diverse range of methods available. However, there are generally two types of scaffolds produced from current fabrication methods, either a fibrous or non-fibrous scaffold. The resulting scaffolds are porous with interconnected pores and controlled pore size and shape.

1.7.1 **Production of non-fibrous scaffolds**

1.7.1.1 **3D printing**

3D printing, or bioprinting is a flexible, automated platform for free-form fabrication of complex living structures of which two, main, distinct technologies have emerged (207). The first of these relies on the use of inkjet printing of either individual or small clusters of cells (208-216). The technique is rapid, versatile and cheap; however, it is difficult to assure the high cell density required for fabrication of solid, organ structures. The
high speed encountered in this technique also causes considerable cellular damage, although recent developments have improved cell survival (207).

The second approach utilises mechanical extruders to place multicellular aggregates of definite composition, also called bioink particles, into a supporting environment using computer-generated templates of the desired biological structure (217-221). The method forms structures by the post-printing fusion of bioink particles and the sorting of cells within the particles. This form of printing also allows cell-cell adhesion within particles providing cells with a more physiologically normal environment. However, the relatively high cost of the printers required for this method are a limiting factor in their use (207).

A study which employed 3D printing demonstrated its effectiveness with the printing of collagen, keratinocytes and fibroblasts to represent the ECM, epidermis and dermis respectively (222). The printed tissue was shown to be morphologically and biologically representative of human skin with keratinocytes undergoing terminal differentiation and forming the stratum corneum, the uppermost layer of epidermis. However, over a 14-day culture period the thickness of the constructs decreased from 500 μm to as thin as 150 μm.

1.7.1.2 Thermally induced phase separation (TIPS)

TIPS has been extensively investigated and produces polymeric structures with controlled porosity and pore size (223). TIPS includes two types of separation, the first of those is liquid-liquid phase separation (224) in which the polymer solution is cooled to produce a polymer-rich phase and polymer-lean phase followed by solvent removal. One such example of this technique was the production of a porous, biocompatible
and biodegradable poly(lactic-co-glycolic acid) (PLGA) scaffolds by dissolving varying concentrations of PLGA in dioxane and water followed by heating, quenching, freezing and solvent evaporation (225).

The second form of separation is solid-liquid phase separation (226), also called lyophilisation. This method is employed in the production of commercial substitutes such as IDRT and MatriDerm with the polymer solution frozen followed by removal of the solvent. Solvent freezing at higher temperatures generally produces larger pores as a low nucleation and crystal growth rate leads to the production of a small number of large solvent crystals (227).

1.7.1.3 Gel casting

Hydrogels have become increasingly prevalent in the last decade (228), likely due to their biocompatibility and structural similarity to the macromolecular-based components in the body (229). They are often considered favourable for promoting cell migration, angiogenesis, high water content and rapid nutrient diffusion (230). According to Jhon and Andrade (1973) there are three different mechanisms of gelling which can be used to form hydrogels: firstly, ionic cross-linking with multivalent counterions is simple but those ions could be exchanged with others in the aqueous environment leading to uncontrolled deterioration of the hydrogels’ original properties (231); secondly, covalent cross-linking provides more precise control but the toxicity of cross-linking molecules and the non-degradable cross-links may not be desirable; and, thirdly, utilisation of the phase transition behaviour of certain polymers (232).

Collagen hydrogels are well studied and show excellent biological functionality (233, 234). For example, a type I collagen hydrogel was fabricated by freeze-drying neutralised collagen followed by immersion in ethanol, evaporation of ethanol, fixation
by UV irradiation and finally addition of cell suspension (235). However, contraction of collagen scaffolds is a major drawback. Volume loss of the gels can be caused by self-compression, contraction, degradation or induced by fibroblasts (236-239). One study has attempted to address this by compressing collagen type I hydrogels prior to cell-seeding (240). It was seen that plastic compression of 5 mL gels to a thickness of 0.5 mm dramatically reduced contraction and degradation while HDFs were still able to evenly distribute throughout the compressed gel without affecting HDF viability or function.

1.7.1.4 **Gas foaming**

Gas foaming involves dissolution of a blowing agent into a hydrogel. When the agent is released this generates a porous scaffold (241, 242). This technique has been used to generate a PCL-thermoplastic gelatin scaffold by gas foaming N$_2$/CO$_2$ through a melt mix of PCL and thermoplastic gelatin and then extracting thermoplastic gelatin by soaking the sample in dH$_2$O (243). Synthetic- and natural-polymer based scaffolds have been produced using this technique (244-246). Previously, polymeric foaming employed organic solvents but supercritical fluids (SCFs) can be used as a good alternative as they eliminate the possibility of leaving unwanted contaminants (247). The advantage of SCFs is that they leave no unwanted contaminants such as residual solvents or salts (248). However, this technique often produces scaffolds too compact for cells (249) and requires specialised equipment which is not readily adapted to most laboratory settings.
1.7.2 Production of fibrous scaffolds

1.7.2.1 Electrospinning

Fibrous scaffolds can be produced by several techniques such as wet spinning (250), needled non-woven or electrospinning. The technique of electrospinning is gaining momentum in the skin tissue engineering field as it is simple and inexpensive (251). A large array of single-polymer and composite scaffolds have been electrospun in skin tissue engineering including synthetic polymers such as PCL (252), polyethylene oxide (PEO) (253), PLA (254) and PGA (255), and natural polymers, such as collagen (172), chitosan (256), silk (257), elastin (258) and hyaluronic acid (259).

In electrospinning, fibres are formed by positively charging a polymer solution as it emerges from a needle and this jet is attracted to a negatively charged or grounded collector. The solvent evaporates as the jet travels towards the collector forming dry, positively-charged, ultrathin fibres which accumulate on the collector to create a non-woven, fibrous mat.

Electrospinning has many advantages including morphological similarity to native ECM by producing nano- to microscale fibres (260) which mimic the local cellular environment (Figure 1.10). These ultrathin fibres provide a large surface area which promotes cell adhesion (260-262). Electrospun scaffolds also provide the appropriate tensile strength required for skin while maintaining scaffold integrity. The internal structure of the scaffolds are highly interconnected which promote gas and fluid exchange and cell infiltration (260, 263).
For example a composite scaffold of poly((D,L-lactide)-co-glycolide) and collagen was electrospun resulting in a scaffold with porosities between 85-90 % and pore sizes ranging from 90-130 μm (264). Enhanced cell attachment, proliferation and ECM secretion were seen to depend on the amount of collagen; however, cell morphology and distribution was similar on all scaffolds containing collagen. Slight cell infiltration was observed on all scaffolds indicating the pore size and scaffold porosity, which were controlled by electrospinning, were the main determinants.

1.8 Importance of scaffold structure on cell-scaffold interactions

Scaffold structure is a strong determinant in the success of a substitute by influencing cell interactions. Macroscopically, a scaffold defines the boundaries for tissue growth with rough, disordered surfaces promoting cell differentiation and ECM synthesis while negatively impacting cell proliferation (265-269). On a microscopic level scaffolds provide a framework for cell attachment, migration and proliferation and ultimately tissue organisation (270) with large pore volumes required for accommodation of a sufficient cell mass for tissue repair (271). Particularly on a microscopic scale high
scaffold porosity and pore interconnectivity are critical to cell infiltration and vascularisation by promoting nutrient and waste exchange, metabolite dispersal and cell signalling. Hindrance of interconnectivity, and therefore inhibition of cell migration, can occur through scaffold degradation creating bottlenecks (272) or through lack of degradation with attached cells blocking pores (271). Pore size also plays an important role by affecting cell proximity during seeding and cell ingrowth with optimal pore sizes varying according to tissue requirements.

1.9 Development of novel scaffolds in skin tissue engineering

There is a plethora of research in skin tissue engineering with many different materials and fabrication methods being investigated using the two major skin cells, i.e. epidermal keratinocytes and dermal fibroblasts. There appears to be a focus on the development of composite scaffolds. This is likely due to their ability to produce scaffolds which encompass the advantageous properties of the individual polymers while overcoming the disadvantages. These combinations consist of all synthetic polymer scaffolds, all natural polymer scaffolds or, more commonly, a blend of synthetic and natural polymers in one scaffold.

An example of a composite scaffold composed entirely of natural polymers is a cross-linked, electrospun, collagen-elastin scaffold (273). It was composed of 20 % bovine, collagen type I and 80 % recombinant, human tropoelastin dissolved in HFP with microscale fibres and 64.5 ± 6.23 % porosity. The scaffold displayed accelerated HDF proliferation compared to pure collagen scaffolds in vitro. When tested as a subcutaneous implant in a mouse model the collagen-elastin scaffold was shown to perform similarly to IDRT with a moderate immune response, HDF infiltration, collagen deposition and angiogenesis observed over 6 weeks.
On the other end of the spectrum, fully synthetic scaffolds have been fabricated via salt leaching (274). Relatively hydrophobic PLA and hydrophilic PEO were used to synthesise two tri-block co-polymers (275), presumably aiming to improve the cell-interactive properties of PLA using PEO’s hydrophilicity. The porous scaffolds were shown to support human fibroblast and keratinocyte proliferation and differentiation over 6 weeks in vitro. Furthermore, the fibroblasts could colonise the scaffold and produce ECM while keratinocytes formed an epidermal barrier.

Another approach to improving the cell-scaffold interactions of synthetic polymers is to fabricate the core of the scaffold with the synthetic polymer and then coat the fibres with natural polymers to provide a favourable surface for cell adherence. This method was used in the fabrication of a PLGA-collagen scaffold in which a PLGA knitted mesh was coated with bovine, collagen type I (206). The PLGA fibre widths were quite large (>100 μm) but the web-like collagen structures in the pores of the PLGA mesh were considered microsponges. The addition of collagen was shown to improve HDF attachment and proliferation in vitro with more homogenous cell and ECM distribution. However, when tested as a living, dermal substitute in a nude, mouse model there was no difference observed between the PLGA and PLGA-collagen scaffolds.

Coating of synthetic fibres has also been performed using a modified version of electrospinning which produced fibres possessing a core-sheath structure. Transmission electron microscope imaging revealed electrospun PCL/silk emulsions formed core-sheath or multicore-sheath structures while maintaining nanoscale fibres (276). The incorporation of silk into the PCL scaffolds was shown to improve HDF attachment and proliferation with increasing silk concentrations improving cell-interactions. These scaffolds were also tested with fluorescein isothiocyanate (FITC) and showed the composite scaffolds had significantly inhibited burst release of FITC
in the first two hours compared to PCL-only scaffolds and then continuous, gradual, long-term release of up to 80 hours. This delayed and prolonged release could be useful during treatment for delivery of drugs or biofactors which could accelerate wound healing.

Blending of synthetic and natural polymers has also been performed by dissolution of all polymers in a single solvent to form one, homogenous solution. Electrospinning of such a solution has been performed using various ratios of bovine collagen and PCL (277). The study chose to use electrospinning as the fabrication method as a previous study had shown pure collagen, electrospun scaffolds significantly decreased wound contraction compared to lyophilised, collagen scaffolds when grafted onto full-thickness wounds in athymic mice (278). The collagen-PCL, electrospun scaffolds were all shown to support cell interactions with decreasing PCL content, particularly PCL concentrations below 10 % (w/v), associated with increasing HDF density, better stratification of dermal and epidermal layers and higher HDF and normal, human, epidermal keratinocytes (NHEK) proliferations rates in vitro indicating PCL may be able to reduce wound contraction. Conversely, decreasing PCL content was seen to result in increased contraction of scaffolds when cultured with HDFs and NHEKs in vitro. Despite these varying results, cell infiltration was observed on all scaffolds by day 10. This indicates the internal structure of scaffolds produced by electrospinning was conducive to cell ingrowth.

1.10 Project rationale and aims

Commercial substitutes provide wound closure and promote repair; however, new skin tissue often lacks the elasticity, strength and complexity of normal skin with scarring and contraction often occurring. Current research in skin tissue engineering is
investigating a wide array of materials and fabrication techniques to improve available treatments.

Elastin is a well-known, ECM protein which plays an important role in the elastic network of skin. This study explores the inclusion of elastin in two, novel scaffolds to determine elastin’s ability to improve current technology, particularly elasticity and performance *in vivo*.

One of the novel scaffolds investigated is a modified version of the commonly used, dermal substitute IDRT in which 10 % (w/w) human, recombinant tropoelastin was incorporated during fabrication to form the final elastin-IDRT (EDRT). The presence of elastin in EDRT is expected to increase elasticity, improve cell-scaffold interactions and accelerate angiogenesis compared to IDRT. These capabilities are critical in a substitute to improve graft take rates and functionality of new skin, especially in full-thickness, burn injuries in which the dermis and all regenerative elements have been lost.

The other scaffold type examined is a novel, electrospun scaffold composed of a mixture of rat tail collagen type I, bovine ligament nuchae elastin and PCL. Electrospinning was chosen for its inherent ability to form scaffolds which closely mimic native ECM by producing highly porous scaffolds with high pore interconnectivity and ultrathin fibres which promote cell attachment, infiltration and fluid exchange. Electrospinning was also chosen as it has been shown to reduce wound contraction compared to lyophilisation. The three polymers were each chosen for advantageous properties they could contribute to the final scaffold. Elastin was chosen for favourable mechanical and cell-interactive abilities, collagen for its natural abundance in ECM and biosignals and PCL for its strength and ability to reduce wound
contraction. These electrospun, triple-polymer scaffolds are the first of their kind produced for use as a skin substitute. As such, early stage testing investigated mechanical and cell-interactive properties with the expectation of improved elasticity and favourable cell attachment and infiltration which, if translated to a clinical setting, could improve regeneration of skin structure, appendages and pliability.

The overall aim of this study was the development of an electrospun scaffold composed of a novel combination of collagen, elastin and PCL and the characterisation of this electrospun scaffold and EDRT in vitro using fibroblasts and keratinocytes and in vivo using established mouse models. This was performed by:

1. characterisation of morphological and mechanical properties of the scaffolds;
2. in vitro characterisation of cell-scaffold interactions by culturing immortalised, human keratinocytes and HDFs on the scaffolds; and
3. investigation of efficacy, ECM deposition, scaffold remodelling and ECM gene expression using established mouse models based on skin excision as burns wounds smaller than 20 % are debrided before treatment.
Chapter 2

Materials and Methods
Chapter 2  Materials and Methods

2.1  Materials

Table 2.1: Table of reagents and consumables

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
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<tbody>
<tr>
<td>Acetic acid, glacial</td>
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2.2 Methods

2.2.1 Development and characterisation of novel, electrospun, triple polymer scaffolds

Electrospun composite scaffolds were developed by varying four parameters; polymer composition, flow rate, air gap and applied electric potential. The effect of each parameter on electrospinning efficiency, scaffold removal, average pore diameter and average fibre width were examined.

2.2.1.1 Fabrication of triple polymer, electrospun scaffolds

Rat tail, collagen type I solution was frozen overnight at -80 °C. Frozen collagen solutions were lyophilised at -45 °C at vacuum. Dry collagen was stored at 4 °C.

Dried collagen, bovine neck ligament elastin and/or PCL were dissolved in HFP. Various polymer compositions (Table 2.1) were tested with total polymer concentration maintained at 15 % (w/v). Additional electrospinning parameters tested were flow rate...
(1, 3 and 5 mL/hr), air gap (10, 15, 20 and 25 cm) and applied electric potential (15-40 kV) (Table 2.1). During electrospinning, 0.5 mL of polymer solution was loaded into a 1 mL syringe (Figure 1A) with a blunt, 18 gauge needle attached. Flow rates were regulated using a single-syringe infusion pump (SP100iZ, World Precision Instruments Inc., FL, USA), air gap was measured by distance between the needle point to the collector and applied electric potential was controlled by transferring a positive charge to the needle (ES30P, Gamma High Voltage Research Inc., FL, USA) and a negative charge to the bronze collector (ES30N, Gamma High Voltage Research Inc., FL, USA) (Figure 2.1). The final, non-woven, porous scaffold was peeled off the collector using fine-tipped forceps.
Table 2.2: Electrospinning conditions tested; collagen, elastin and PCL.

<table>
<thead>
<tr>
<th>Solution composition (% (w/v))</th>
<th>Flow rate (mL/h)</th>
<th>Air gap (cm)</th>
<th>Applied electric potential (kV)</th>
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<tr>
<td>7.5 % collagen 7.5 % PCL</td>
<td>1 10</td>
<td>25</td>
<td></td>
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<td></td>
<td>1 20</td>
<td>30</td>
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</tr>
<tr>
<td></td>
<td>3 25</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>3 % collagen 12 % elastin</td>
<td>1 10</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 15</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 20</td>
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<td></td>
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<td></td>
<td>3 15</td>
<td>20</td>
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</tr>
<tr>
<td></td>
<td>3 20</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 15</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>1.5 % collagen 12 % elastin 1.5 % PCL</td>
<td>1 10</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 15</td>
<td>20</td>
<td></td>
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<td></td>
<td>1 20</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 15</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 20</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>2 % collagen 12 % elastin 1 % PCL</td>
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<td></td>
<td>1 20</td>
<td>20</td>
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<tr>
<td></td>
<td>1 25</td>
<td>20</td>
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<td></td>
<td>3 20</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 25</td>
<td>30</td>
<td></td>
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<tr>
<td>2.5 % collagen 12 % elastin 0.5 % PCL</td>
<td>1 15</td>
<td>20</td>
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<tr>
<td></td>
<td>1 20</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>3 20</td>
<td>20</td>
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</tbody>
</table>
Figure 2.1: Schematic of electrospinning showing the three main stages; jet emergence (A), instability (B) and fibre collection (C). Adapted from (279).
2.2.1.1 *Cross-linking of electrospun scaffolds*

The collagen and elastin polymers used are water soluble, so cross-linking was used to preserve scaffold structure. The triple polymer scaffolds were cross-linked using 25 % (v/v) glutaraldehyde (1 mL) (Sigma-Aldrich) vapour cross-linking (273) for 24 hrs at room temperature (RT). After 24 hrs, cross-linking was stopped by immersing scaffolds in 0.2 M glycine overnight to quench any unreacted glutaraldehyde. Cross-linked scaffolds were then washed in PBS to remove glycine, sterilised in 80 % (v/v) ethanol for 24 hrs and washed in 3 × sterile PBS.

2.2.1.2 *Structural analysis of electrospun scaffolds using SEM analysis*

Non-cross-linked and cross-linked electrospun scaffolds were dehydrated by lyophilisation. Dry samples were mounted on aluminium sample stubs and sputter-coated with platinum using an auto coater (JFC-1600 Auto Fine Coater, JEOL Ltd., Tokyo, Japan) prior to examination by SEM (JEOL JSM-6380, JEOL Ltd., Tokyo, Japan) at a voltage of 15 kV.

Pore diameter and fibre width of scaffolds were quantified using SEM images. Pore diameter was defined as the longest axis of a pore (273) (Figure 2.2, blue lines) and fibre widths were measured at cross-sections of individuals fibres (Figure 2.2, yellow lines). Three images were taken per sample and 10 pore diameters and 30 fibre widths were randomly chosen from each image and measured using ImageJ (National Institute of Health) for a total measurement of 30 pore diameters and 90 fibre widths per sample.
Figure 2.2: SEM image of non-cross-linked (A) and cross-linked (B), electrospun, CEP scaffold demonstrating pore diameter (blue lines) and fibre width measurements (yellow lines).

Four cross-linked, triple polymer, electrospun scaffolds and two, control, double-polymer scaffolds (Table 2.2) were chosen for uniaxial tensile test based on pore diameter and fibre width results. Henceforth, all electrospun scaffolds referred to are cross-linked unless otherwise specified.

Table 2.3: Electrospinning conditions of scaffolds chosen for uniaxial tensile tests; collagen-PCL scaffold (CP), collagen-elastin scaffold (CE) and collagen-elastin-PCL scaffold (CEP).

<table>
<thead>
<tr>
<th>Scaffold</th>
<th>Solution composition (% (w/v))</th>
<th>Flow rate (mL/h)</th>
<th>Air gap (cm)</th>
<th>Applied electric potential (kV)</th>
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</thead>
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<tr>
<td>CP</td>
<td>7.5 % collagen 7.5 % PCL</td>
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<td>40</td>
</tr>
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<td>CE</td>
<td>3 % collagen 12 % elastin</td>
<td>3</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
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<td>1.5 % collagen 12 % elastin 1.5 % PCL</td>
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<td>15</td>
<td>25</td>
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<td>CEP 2</td>
<td>2 % collagen 12 % elastin 1 % PCL</td>
<td>1</td>
<td>20</td>
<td>20</td>
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</tbody>
</table>
### Uniaxial tensile tests of electrospun scaffolds

Uniaxial tensile tests allow determination of the mechanical strain – stress relationship and modulus of scaffolds. The mechanical strain, $\varepsilon$, can be defined and calculated as

$$\varepsilon = \frac{L - L_0}{L_0}$$

where $L$ is the deformed length and $L_0$ is the original length of the specimen. While strain does not have unit, it may read as mm/mm or percent (%) for convention in biomedical engineering.

Strips of cross-linked, electrospun scaffolds were used for tensile tests. The uniaxial elongation tensile tests were performed in an unconfined state using an Instron (Model 5543) with a 10 N biological load cell (Figure 2.3). All tests were run in PBS at 37 °C. Prior to mechanical testing, the samples were soaked for at least 2 hrs in PBS at 37 °C to yield isotonic hydrogels. The thickness (0.88 ± 0.01 mm), width (9.05 ± 0.05 mm) and height (16.28 ± 1.02 mm) prior to the tests.

For the uniaxial tensile tests, the samples were clamped as seen in Figure 2.3 and subjected to a loading and unloading cycle at 37 °C [16] and the load (N) was applied at a cross speed of 0.5 mm/min. From the tensile tests, the ultimate strain to rupture was found to be 40 % for the scaffold samples.

Note that the uniaxial tests of scaffolds exhibit a non-linear relationship between strain and stress (Figure 2.4). For this reason, the compressive and tensile Young’s modulus was obtained approximately from the tangent slope of the stress-strain curve between 0.05 mm/mm and 0.10 mm/mm strain. For all the samples, the energy loss from the loading
to the unloading process was computed in the strain range of 0-0.15 mm/mm in the tensile tests.

Figure 2.3: Image of a CEP, electrospun scaffold specimen which has undergone uniaxial tensile testing.
Figure 2.4: Representative schematic of a non-linear relationship between stress and strain.

2.2.1.4 Quantification of porosity

Punch biopsies (8 mm in diameter) of CEPs were fixed in 10 % (v/v), neutral-buffered formalin for 24 hrs followed by dehydration in 70 % (v/v) ethanol for 24 hrs, 2 × 1 hr 70 % (v/v) ethanol, 5 × 1 hr 100 % ethanol and 3 × 1 hr xylene incubations. Fixed and dehydrated CEPs were soaked in 3 changes of paraffin for 1 hr each and embedded in paraffin. Cross-sections (4 μm) were cut and mounted on SuperFrost Plus slides, followed by deparaffinisation in 3 × 2 mins xylene incubations, xylene removal in 100 % ethanol (3 × 2 mins) and rehydration in tap water for 2 mins. Sections were incubated in Harris’ Haematoxylin for 5 mins, which stains cell nuclei purple, and excess haematoxylin was removed in tap water. Differentiation was performed using acid alcohol (0.3 % (v/v) HCl in 70 % (v/v) ethanol) followed by a 4 min tap water wash. Finally, a 30 sec incubation in alcoholic eosin stained soft tissue bright pink and excess solution was removed in tap water. Samples were dehydrated with 100 % ethanol
(3 × 1 min) followed by 2 xylene washes. Stained sections were imaged with a bright field microscope (EVOS FL Auto Cell Imaging System, Thermo Fisher Scientific, MA, USA) and colour images were converted to binary images using ImageJ in which fibres appeared black and interfibre spaces white (Figure 2.5). The porosity was calculated using ImageJ and defined as the percentage of interfibre space relative to the total scaffold area measured.

![Figure 2.5: Example of H&E image of CEP cross-section (A) converted to a binary image (B) using ImageJ for quantification of porosity.](image)

### 2.2.1.5 Preparation of CEP 1 and CEP 2 for cell culture studies

CEP samples were sterilised in 80 % (v/v) ethanol for 24 hrs and washed with sterile 3 changes of PBS. Sterilised CEPs were partly dried by quickly touching wet scaffolds to sterilised filter paper and then placed in sterile container for immediate seeding of cells.

### 2.2.1.6 Culture of CEP 1 and CEP 2 with human keratinocytes

HaCaT cells (gifted from the Weiss lab at The University of Sydney) are an immortalised keratinocyte cell line used as a substitute for NHEKs (280-282). Although
HaCaTs are cytogenetically abnormal, they are capable of differentiation under *in vitro* and *in vivo* conditions to form epidermal-like structures (283-285).

**2.2.1.6.1 Maintenance of HaCaTs**

HaCaTs were maintained in HaCaT media prepared with 10 % (v/v) FBS, 2 mM L-Glutamine, 100 U penicillin-0.1 mg streptomycin-0.2 mg neomycin/mL in DMEM. HaCaT cells were cultured and expanded in a static cell culture system at 37 °C and 5 % CO₂ (3111 Forma Series II Water-Jackets CO₂ Incubator, Thermo Scientific, MA, USA).

HaCaTs were detached from tissue culture flasks when approximately 90 % confluency was obtained for sub-culturing, cryopreservation or experiments. Media was removed from flasks and attached cells were rinsed with PBS. HaCaTs were detached by incubating with 5 mL of 0.25 % (w/v) trypsin-0.02 % (w/v) EDTA for 5 mins at 37 °C. Enzymatic treatment was stopped by adding 5 mL pre-warmed HaCaT media to inhibit trypsin activity. The cell suspension was then centrifuged at 1000 rpm/min for 10 mins to pellet live HaCaTs. After the supernatant was discarded, live HaCaTs were resuspended in pre-warmed HaCaT media, split 1:2 or 1:3 and transferred to new culture flasks. Cells were incubated at 37 °C and 5 % CO₂ and media was replaced every 2-3 days.

**2.2.1.6.2 Cryogenic storage of HaCaTs**

After cell detachment, HaCaTs were resuspended in HaCaT media containing 10 % (w/v) DMSO, a cryoprotectant. The HaCaT cell suspension, at a concentration of approximately 1 × 10⁶ cells/mL in cryovials, were placed in a cryogenic cooling container (Mr Frosty, Thermo Fisher Scientific, MA, USA), containing isopropanol
(VWR) and frozen at -1 °C/min overnight to -80 °C and stored at -80 °C for up to 1 month or transferred to liquid nitrogen for long-term storage.

2.2.1.6.3 Retrieval of cryopreserved HaCaTs

Cryopreserved HaCaTs were thawed at room temperature (RT) and immediately transferred to sterile, cell culture flasks containing pre-warmed HaCaT media. HaCaT cells were left to attach overnight and the media replaced with normal, HaCaT media within 24 hrs to remove residual DMSO.

2.2.1.6.4 Quantification of viable HaCaTs by Trypan Blue staining

Detached HaCaT suspensions (15 μL) were diluted with an equal volume of 0.4 % (w/v) Trypan Blue solution and cell number was quantified using a haemocytometer (Hemocytometer, Weber Scientific, NJ, USA). Briefly, 10 μL of suspension was placed onto each side of the haemocytometer and only the live cells counted in the 4 corner squares and centre square of both chambers (ten squares total). Dead cells were distinguished by their blue appearance as live cells had an intact cell membrane which excluded the dye.

Original cell density was calculated using the formula below, in which 2 is the dilution factor from addition of the Trypan Blue solution and $x$ is the dilution factor, if any, of the original cell suspension. The resulting cell number had the unit cells/mL.

$$\text{cell density} = \text{cell count} \times 10^3 \times 2 \times x,$$

2.2.1.6.5 Cell culture of HaCaTs on CEPs

HaCaTs were droplet-seeded at a density of $5 \times 10^5$ cells/20 μL onto punch biopsies (8 mm in diameter) of CEP 1 and CEP 2, giving a final cell number of $1 \times 10^6$ cells/cm² on the scaffolds. HaCaTs were left to attach to the scaffolds for 30-60 mins. During
cell attachment, scaffolds remained in cell culture media. Pre-warmed HaCaT media was gradually added to the edge of the wells to minimise disturbance of attached cells. HaCaT media was added to a total of 600 μL and replaced with fresh media every 2-3 days. Cell-seeded scaffolds were incubated at 37 °C and 5 % CO₂ in a static cell culture system and samples were collected on days 1, 14 and 28 post-seeding for histological analysis.

2.2.1.7  **Culture of CEP 1 and CEP 2 with HDFs**

HDFs were isolated from donated skin biopsies of patients at the Burns Unit in Concord Repatriation General Hospital with approval from the Hospital Human Ethics Committee (Ethics number: CH 62/6/2006-026). HDF media consisted of DMEM, 100 U penicillin-0.1 mg streptomycin-0.2 mg neomycin/mL and 10 % (v/v) FBS.

2.2.1.7.1  **Maintenance, cryopreservation and retrieval of HDFs**

HDFs were maintained, cryopreserved and retrieved according to HaCaT protocols in sections 2.2.1.6.1-2.2.1.6.3 using HDF media.

2.2.1.7.2  **Cell culture of HDFs on CEPs**

Similar to HaCaT seeding, HDFs were droplet-seeded with $5 \times 10^4$ cells/20 μL onto punch biopsies (8 mm in diameter) of CEP 1 and CEP 2 for a final cell concentration of $1 \times 10^5$ cells/cm² per scaffold. HDFs were left to attach for 30-60 mins and then 600 μL of pre-warmed HDF media was added. Scaffolds were kept immersed in media using a 100 μL pipette tip and media was changed once a day. Cell-seeded scaffolds were incubated at 37 °C and 5 % CO₂ in a static cell culture system and were collected on days 1, 14 and 28 post-seeding for histological analysis.
2.2.1.8 *Assessment of seeding efficiency and proliferation of HaCaTs using the MTS assay*

In this assay, there is a direct relationship between absorbance and the number of metabolically active cells as only viable cells convert 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, innersalt (MTS) to a soluble, coloured formazan product by dehydrogenase enzymes and an electron coupling reagent, phenazine methosulfate (PMS). Therefore, a standard curve was first produced to plot the relationship between absorbance. HaCaT concentrations of 1.0, 2.5, 5.0, 7.5, 10.0, 25.0 and 50.0 × 10^3 cells/well were used.

Cell-seeding efficiency was examined on day 1. All cell-seeded CEPs were moved from the culture plate to a new plate, washed and re-immersed in fresh media. HaCaTs attached to the original culture dish were washed with new media followed by incubation in 200 μL HaCaT media with 40 μL of MTS/PMS reagent. The incubation was performed for 1 hr at 37 °C and 5 % CO₂ in the dark. The media was transferred to a 96 well plate and the absorbance read at 490 nm. Cell number was calculated from the absorbance using the standard curve. Cell-seeding efficiency of HaCaTs was calculated using the following formula and expressed as a percentage of the seeded number of cells.

\[
\text{HaCaT-seeding efficiency} = \frac{\text{HaCaT number}}{500,000} \times 100
\]

Quantification of HaCaT numbers was performed on days 7, 14, 21 and 28 post-seeding and an unseeded scaffold was used as a control for possible protein interference. Cell-seeded scaffolds were transferred to new plates and washed with fresh media followed by incubation with 200 μL HaCaT media and 40 μL MTS/PMS reagent as described above and the absorbance was used to calculate the HaCaT
Detachment of HaCaTs from the scaffold was not required as all metabolically active scaffolds, whether located in the interior or on the exterior of the scaffold, converted MTS to the coloured, formazan product.

2.2.1.9 Assessment of seeding efficiency and proliferation of HDFs using the MTS assay

A standard curve for HDFs was produced using HDF concentrations of 1.0, 2.5, 5.0, 7.5, 10.0 and 25.0 × 10^3 cells/well. Cell-seeding efficiency was examined on day 1 as described above for examination of HaCaT-seeding efficiency in section 2.2.1.8. Cell-seeding efficiency of HDFs was calculated using the following formula and expressed as a percentage of the seeded number of cells.

\[
\text{HDF-seeding efficiency} = \frac{\text{HDF number}}{50,000} \times 100
\]

Quantification of HDF numbers was performed on days 7, 14, 21 and 28 post-seeding as described above for quantification of HaCaT proliferation in section 2.2.1.8.

2.2.1.10 Co-culture of HaCaTs and HDFs on CEP 1 and CEP 2

In the study both HaCaTs and HDFs were cultured on the same side of the same scaffold (Figure 2.6). Cell-seeded scaffolds were incubated at 37 °C and 5 % CO₂ in a static cell culture system. Samples were collected on days 1, 14 and 28 post-seeding for further analysis.
2.2.1.10.1 *Air-liquid interface co-culture of HaCaTs and HDFs on CEP 1 and CEP 2*

Punch biopsies of CEP 1 and CEP 2 (8 mm in diameter) were placed on top of sterile gauze and HDFs were seeded and cultured as described in section 2.2.1.7.2 over 7 days (Figure 2.6, steps 1 and 2). After 7 days, HaCaTs were seeded onto the same surface as HDFs (Figure 2.6, step 3) as described in section 2.2.1.6.5. HaCaT media was added until it contacted the base of the scaffold while the surface of the scaffold was exposed to air (Figure 2.6, step 4). The seeded scaffolds were then incubated at 37 °C and 5 % CO₂ in a static culture system and monitored to ensure the media was in contact with the scaffold. Media was replaced every 2-3 days.

*Figure 2.6: Schematic of air-liquid interface cell-seeding and culture.*
2.2.1.11 **SEM of cell-seeded CEP 1 and CEP 2**

Cell-seeded scaffolds were collected for SEM analysis on days 1, 14 and 28. Samples were washed in sterile PBS twice, then fixed with 2.5 % (w/v) glutaraldehyde (ProSciTech) for 2 hrs and washed 3 × 2 mins in 0.1 M cacodylate buffer. Samples were then immersed in 2 % OsO₄ in 0.1 M cacodylate buffer for a secondary fixation followed by washing twice with 0.1 M cacodylate buffer. Fixed samples were dehydrated with increasing concentrations of ethanol (2 mins each of 1 × 50 % (v/v), 3 × 70 % (v/v), 3 × 90 (v/v) %, 2 × 100 % and 2 × 100 % (molecular sieve)) followed by chemically drying with HMDS. Scaffolds were mounted on aluminium sample stubs, sputter-coated with platinum and examined by SEM.

2.2.1.12 **H&E analysis of cell-seeded CEP 1 and CEP 2**

Cell-seeded scaffolds were collected for H&E staining on days 1, 14 and 28. Samples were fixed in 10 % (v/v), neutral-buffered formalin for 24 hrs followed by dehydration in 70 % (v/v) ethanol for 24 hrs. Further dehydration was performed with two 1 hr incubations in 70 % (v/v) ethanol, five 1 hr incubations in 100 % ethanol and then 3 × 1 hr incubations in xylene. Scaffolds were then soaked in 3 changes of paraffin for 1 hr and 20 mins each, embedded in paraffin blocks and cross-sections (4 μm) were mounted on SuperFrost Plus slides.

H&E staining was performed to visualise cell distribution in scaffolds. Cross-sections were deparaffinised in 3 changes of xylene for 2 mins each followed by 3 × 2 min 100 % ethanol incubations to remove xylene. Sections were then rehydrated in tap water for 2 mins. Samples were incubated in Harris’ Haematoxylin for 5 mins, which stains nucleic material purple, and excess haematoxylin was removed by washing in tap water until clear. Differentiation was performed using acid alcohol (0.3 % (v/v) HCl
in 70 % (v/v) ethanol) to ensure good chromatin detail followed by a 4 min wash in tap water. A 30 sec incubation in alcoholic eosin stained tissue bright pink and excess solution was removed with a 2 min wash in tap water. Samples were dehydrated in $3 \times 1$ min 100 % ethanol incubations followed by 2 xylene washes. Sections were then coverslipped and imaged using a brightfield microscope (Olympus BX60, Olympus, PA, USA).

2.2.1.13 Fluorescence analysis of cell-seeded CEP 1 and CEP 2

Cell-seeded CEP scaffolds were collected on days 1, 14 and 28. Cell-seeded scaffolds were rinsed with sterile PBS and fixed in 4 % (v/v) paraformaldehyde for 15 mins at RT. Samples were then immersed in 0.2 % (v/v) Triton-X for 15 mins to permeabilise the cell membrane and rinsed in sterile PBS $3 \times 5$ mins. Fixed, HDF-seeded scaffolds were then embedded in OCT compound and frozen in 100 % ethanol chilled with dry ice. Sections (10 μm) were cut by cryosectioning (Leica CM 3050 S, Leica, NSW, Australia) and stained with Slow Fade Gold antifade reagent containing DAPI overnight in the dark followed by imaging using a fluorescent microscope (EVOS, FL Auto Cell Imaging System, Thermo Fisher Scientific, MA, USA).

2.2.2 Characterisation of novel EDRT

Single-layer, silicone-free IDRT was used for all studies throughout this thesis.

Elastin-modified IDRT, EDRT, was provided by Elastagen.
2.2.2.1 *Structural analysis of EDRT and IDRT using SEM analysis*

Sections of EDRT and IDRT (1 × 1 cm²) were cut using a scalpel blade followed by washing in PBS for 5 mins and then 3 × 5 mins washes of MilliQ water. Both EDRT and IDRT were lyophilised overnight at -45 °C at vacuum and mounted onto aluminium sample stubs. Samples were sputter-coated with platinum and examined by SEM as described in section 2.2.1.2.

Pore diameters of EDRT and IDRT were quantified using SEM images. Pore diameter was defined as the longest axis of a pore (273) and measured using ImageJ as described in section 2.2.1.2. Thirty pores were measured for EDRT and IDRT each. In addition, porosity was analysed by converting H&E images to binary images as described in section 2.2.1.4.

2.2.2.2 *Uniaxial compression tests of EDRT and IDRT*

Uniaxial compression tests were utilised to assess the mechanical property of EDRT and IDRT. The compression tests were performed in an unconfined state using a Series 5540 Single Column System - 5543 (Instron, Melbourne, VIC, Australia) with a 10 N biological load cell according to the testing procedure (286, 287). Prior to this mechanical testing, the samples were soaked for at least 2 hrs in PBS. The thickness (1.0 ± 0.1 mm) and dimensions (10.0 × 10.0 mm) of each sample were then measured using digital callipers. The compressive properties of the samples were tested in PBS at 37 °C.

During the test, samples were placed between two plates (Figure 2.7) and subjected to a single loading and unloading cycle. The compressive load (N) (288) was applied at a cross speed of 0.5 mm/min and up to a 40 % final strain level. The compressive
Young’s modulus was obtained as the tangent slope of the stress-strain curve between 5-10 % strain level. Also, energy loss, based on the compression cycle, was computed for all samples.

Figure 2.7: Image of equipment used for compression tests.

2.2.2.3 **Co-culture of HaCaTs and HDFs on EDRT and IDRT**

Sections of EDRT and IDRT (1 × 1 cm²) were cut using a scalpel blade and then sterilised in 80 % (v/v) ethanol for 24 hrs followed by 3 washes in sterile PBS under sterile conditions. Before cell-seeding, sterilised scaffold sections were partly dried by briefly touching wet scaffolds to sterilised filter paper to partly absorb PBS held in the scaffolds. Scaffolds were cultured with cells as described in section 2.2.1.6.5 for HaCaTs and section 2.2.1.7.2 for HDFs.
2.2.2.4 Assessment of HaCaT- and HDF-seeding efficiency and HaCaT proliferation on EDRT and IDRT using the MTS assay

Cell-seeding efficiency was examined and calculated as previously described in section 2.2.1.8 for HaCaTs and section 2.2.1.9 for HDFs. HaCaT proliferation was assessed as described in section 2.2.1.8.

2.2.2.5 Assessment of HDF proliferation on EDRT and IDRT by haemocytometer

The number of HDFs was quantified on days 7, 14, 21 and 28 post-seeding using a haemocytometer (Weber Scientific, NJ, USA). HDF-seeded scaffolds were transferred and washed with sterile PBS. Cells were detached from the scaffolds using 500 μL of 0.25 % (w/v) trypsin-0.02 % (w/v) EDTA per well followed by incubation at 37 °C and 5 % CO₂ for 5 mins. The number of HDFs detached from EDRT or IDRT was counted using a haemocytometer as described in section 2.2.1.6.4.

2.2.2.6 Analysis of HaCaT-seeded and co-cultured EDRT and IDRT by SEM and H&E staining

Samples of HaCaT-seeded and co-cultured scaffolds for SEM analysis were collected on days 1, 14 and 28 post-seeding and imaged as described in section 2.2.1.11. Samples for histological analysis were H&E stained as described in section 2.2.1.13.

2.2.2.7 Fluorescence analysis of cell-seeded EDRT and IDRT

HDF-seeded scaffolds were collected on days 1, 14 and 28 or 7, 14 and 21 post-seeding and imaged as described in section 2.2.1.13.
2.2.2.8 Assessment of HDF-induced EDRT and IDRT contraction

HDF-seeded EDRT and IDRT were photographed on day 0. Cell-seeded scaffolds were then photographed with two day intervals over 28 days. The size of scaffolds was then measured using ImageJ. Scaffold contraction was expressed as a percentage of the original size and calculated using the following formula.

\[ \text{contraction (\%)} = \frac{\text{current size}}{\text{original size}} \]

2.2.3 Animal studies of novel, skin substitutes

2.2.3.1 Source of mice and ethics approval

Mice were purchased from the Australian Animal Centre (WA, Australia) under ethics number AWC No. 2013/019.

2.2.3.2 Number of mice and gender distribution

There were four mice per group to ensure results were in triplicate and to account for possible variations in wound healing due to gender at least one male or female was included in each group.

2.2.3.3 Preparation of scaffolds for grafting in mice

Punch biopsies (8 mm in diameter) of CEP 1, CEP 2, EDRT and IDRT were sterilised in 80 % (v/v) ethanol for 24 hrs and washed 3 times with sterile PBS in a sterile environment. Sterilised scaffolds in sterile PBS were stored and transported in specimen containers.
2.2.3.4 **Surgery**

Pathogen-free, female (22.0 ± 1.6 g) and male (26.0 ± 1.8 g), Balb/c mice, aged 12 weeks were housed in cages under standard conditions (19-22 °C, 12 hr light and 12 hr dark cycle) with free access to food and water.

Before surgery, each mouse was individually anaesthetised by intra-peritoneal injection of a mixture of ketamine (75 mg/mL) and xylazine (10 mg/mL) at 0.01 mL/g of body weight. Anaesthesia was monitored throughout the procedure by testing the rear foot reflex and eyes were kept moist with sterile saline.

2.2.3.5 **Subcutaneous implantation of CEP 1, CEP 2, EDRT and IDRT in mice**

A subcutaneous implant, mouse model was chosen as this type of model is effective for screening samples and has previously been used for examining host responses such as cell-scaffold interactions and inflammation (273).

The dorsal area of the mice was shaved (WAHL Specialty Rodent Hair Clipper, Able Scientific, WA, Australia) and cleaned with Betadine solution (Sanofi-Aventis Consumer Healthcare, QLD, Australia) and sterile saline. Four, 1 cm long incisions were made on the dorsal area separated by normal skin (Figure 2.7). Subcutaneous pouches were created using surgical scissors into which either CEP 1, CEP 2, EDRT or IDRT were placed according to implant patterns (Figure 2.7). All scaffolds were sutured to the skin before closing incisions with sutures. Wounds were dressed with a non-sticky dressing, Atrauman, and a sticky dressing, IV3000, which were maintained for the first 7 days post-surgery. Carprofen (5 mg/kg) was injected at the time of anaesthesia and for the following two days post-surgery as analgesia.
After surgery, each mouse was caged individually post-surgery for the first two days and then two mice per cage thereafter with free access to food and water. Wound dressings were checked every day for the first 7 days. Mice were weighed on days 1-7, 9, 11, 14 and 21 post-surgery and if a mouse lost more than 10% of its original body weight the mouse was euthanised. Mice were also monitored for normal grooming and behaviour, if scruffy appearance or frightened behaviour was noticed the mouse was housed separately for the remainder of the study. Mice were sacrificed using CO₂ on weeks 1, 2, 4, 12 and 24 post-surgery and skin biopsies were collected for histological, immunohistochemical (IHC) and gene expression analysis.

Figure 2.8: Schematic of CEP 1, CEP 2, EDRT and IDRT subcutaneous implant pattern in mice.
2.2.3.6 **Histological analysis of implants using H&E and Masson’s Trichrome staining**

Skin biopsies of wounds implanted with either CEP 1, CEP 2, EDRT or IDRT were processed, sectioned (4 μm) and stained by H&E as described in section 2.2.1.12. Cross-sections were also stained by Masson’s Trichrome to visualise collagen fibres. Sections were deparaffinised in xylene 3 × 2 mins followed by xylene removal in 100 % ethanol 3 × 2 mins and rehydration in tap water for 2 mins. Samples were then incubated in Bouin’s Fluid for 1 hr at 56 °C and excess solution was removed by rinsing with tap water until clear. The second fixation in Bouin’s Fluid allows for crisper, nuclear staining. Nuclei were then double stained with Celestine Blue (5 % (w/v) ammonium iron (III) sulphate dodecahydrate, 1 % (w/v) celestin blue B, 14 % (v/v) glycerin and 0.1 % (v/v) sulphuric acid) for 5 mins at RT followed by immediate staining with Harris’ Haematoxylin for 5 mins at RT. Excess solution was rinsed away until tap water was clear. Initial differentiation for clear chromatin detail was performed with a brief dip in acid alcohol (1 % (v/v) HCl in 70 % (v/v) ethanol) immediately followed by a 4 min wash in tap water. Muscle was stained red in Ponceau-Acid Fuchsin solution (0.05 % (w/v) acid fuchsin, 0.05 % (w/v) xylidine ponceau and 1 % (v/v) glacial acetic acid) for 10 mins and then rinsed until tap water ran clear. A second differentiation step to decolourise collagen fibres was performed with Phosphomolydic- Phosphotungstic acid (2.5 % (w/v) phosphomolybdic acid and 2.5 % (w/v) phosphotungstic acid) for a minimum of 5 mins or until fibres were colourless. Once fibres were colourless, samples were directly immersed in Light Green solution (1 % (w/v) light green SF yellowish and 1 % (v/v) glacial acetic acid) for 10 mins followed by a 1 min wash in tap water. Stained samples were dehydrated with 3 × 1 min 100 % ethanol incubations followed by two xylene washes and then air-
dried. Dry slides were coverslipped and imaged with a brightfield microscope (Olympus BX60, Olympus, PA, USA).

2.2.3.7 **IHC staining**

Sections were deparaffinised in xylene 3 × 2 mins and then xylene removed with 3 × 2 min 100 % ethanol incubations. Rehydration was performed by immersing slides in tap water for 2 mins followed by boiling slides in fresh 0.01 M citrate buffer (pH 6.0) for 5 mins. The citrate buffer breaks protein cross-links unmasking antigens and epitopes to enhance staining intensity of antibodies. Slides were then washed in PBT buffer containing (0.015 % (w/v) bovine serum albumin, 0.01 % (v/v) Triton X-100 in PBS). Samples were incubated with 3 % hydrogen peroxide for 5 mins to block endogenous peroxidase activity and washed with PBT buffer for 2 mins. The sections were incubated with 5 % (v/v) goat serum (VECTASTAIN Elite ABC Kit) for 30 mins to prevent non-specific binding of the secondary antibody to the sample. Excess goat serum was removed with PBT buffer washes. Primary antibodies at the specified dilutions (Collagen type I 1/500, Elastin 1/300, CD146 1/500) were incubated with the sample overnight at 4 °C and unbound primary antibody was removed. The secondary rabbit antibody was incubated with the samples at a dilution of 1/200 for 1 hr at RT and the unbound secondary antibody was removed with PBT buffer. ABC reagent (made 30 mins prior to use) was added for 30 mins then removed by washing with PBT buffer. Visualisation of fibres with bound primary antibody was performed using diaminobenzidine chromogenic (DAB) substrate (DAB reagent from DAB Substrate kit; made 10 mins prior to use) with 60 secs exposure, then counterstaining with Harris’ Haematoxylin for 30 secs, followed by dehydration with 3 × 1 min 100 % ethanol
incubations and 2 xylene washes. All samples were imaged with a brightfield microscope (Olympus BX60, Olympus, PA, USA).

2.2.3.8 **Gene expression analysis of implanted CEP 1, CEP 2, EDRT and IDRT skin biopsies**

2.2.3.8.1 **Sample collection and processing to preserve mRNA**

Implanted CEP 1, CEP 2, EDRT and IDRT skin biopsies were collected weeks 1, 2, 4, 12 and 24 post-surgery and placed in sterile, DNase/RNase free tubes. Samples were frozen in liquid nitrogen and at -80 °C until messenger RNA (mRNA) isolation.

2.2.3.8.2 **Isolation and clean-up of mRNA from skin biopsies**

mRNA isolation was performed using sterile equipment and consumables. Frozen skin biopsies were cut into small pieces and transferred to 5 mL tubes containing 1 mL cold TRIzol reagent. Skin was then disrupted by alternately homogenising (T10 basic S5 Ultra-Turrax Disperser, IKA, Selangor, Malaysia) at setting 6 (approximately 30,000 rpm) for 30 secs and placing on ice for 30 secs until skin biopsies were well homogenised with no skin pieces visible. The homogenate was transferred to a new 1.5 mL microfuge tube, incubated at RT for 5 mins after which 200 μL of chloroform was used to extract the proteins from the aqueous phase into the organic phase. The mixture was centrifuged at 12,000 g for 15 mins at 4 °C to form 3 phases. The top layer contained the RNA and was carefully transferred to a new 1.5 mL, DNase/RNase free tube without disrupting the protein interphase and 500 μL of isopropanol (Sigma) was added to precipitate RNA. The mixture was repeatedly inverted and then incubated at RT for 10 mins on a plate shaker. Centrifugation at 12,000 g for 10 mins at 4 °C formed a pellet of RNA at the bottom of the tube. The supernatant was
discarded and the RNA pellet was washed by vortexing in 1 mL of 80 % (v/v) ethanol and centrifuging at 7,500 g for 5 mins at 4 °C. The supernatant was discarded and the wash repeated. The clean pellet was left to air dry for 3-5 mins before redissolving in 100 μL of DNase/RNase free water. The sample was then cleaned using the NucleoSpin RNA Clean-up kit (Macherey-Nagel, Germany) following the manufacturer’s protocol then checked for concentration and purity or stored at -80 °C.

2.2.3.8.3 Contamination check and determination of concentration of isolated mRNA

Purity of the isolated mRNA was examined for contamination by protein or organic compounds using absorbance ratios. Nucleic acids (i.e. RNA and DNA) absorb at 260 nm due to the aromatic base present in their structures. Since aromatic proteins (e.g. tryptophan, phenylalanine, tyrosine and histidine) absorb at 280 nm an absorbance ratio between 260nm/280nm (A260/A280) lower than 1.8 indicates protein contamination. Organic compounds have strong absorbance around 230 nm, therefore, an A260/A230 ratio lower than 1.8 indicates contamination by organic compounds. mRNA concentration was also determined; at an optical density of 1.0 RNA has a concentration of 40 μg/mL. Using this, mRNA concentration was calculated using the formula \( \text{mRNA (μg/mL)} = \frac{OD \times 40}{\text{OD}} \) on a UV/Visible Spectrophotometer (Biochrom WPA Lightwave II UV/Visible Spectrophotometer, Biochrom, Cambridge, UK) which calculates A260/A280, A260/A230 and mRNA concentration using the Nucleic Acids and RNA setting.

2.2.3.8.4 mRNA degradation check of isolated mRNA

RNA samples were checked for degradation using gel electrophoresis. Isolated mRNA samples were run on a 1 % (w/v) agarose gel containing 0.05 % (v/v) SYBR Safe in
1× TBE buffer (0.089 M tris base, 0.089 M borate and 0.002 M EDTA, pH 8.3). 2 μg of each mRNA sample mixed with 8 μg of 1× loading dye (5× DNA loading buffer blue diluted 1 in 5 with MilliQ) were run at 90 V for at least 30 mins or until the loading dye had reached the base of the gel. The gel was then imaged (ChemiDoc MP System, Bio-Rad, CA, USA) and Hyperladder IV was used for size reference. mRNA integrity for each sample was deemed acceptable if two clear bands were visible.

2.2.3.8.5 Complementary DNA (cDNA) synthesis by reverse transcription of mRNA

mRNA was converted into cDNA using the SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen, VIC, Australia) following the provided protocol. No Template Controls were included to check for DNA contamination of samples. cDNA was diluted to a final concentration of 10 ng/µL and aliquoted into 20 µL for storage at -20 °C.

2.2.3.8.6 Primer design for genes of interest; collagen type I and elastin

The forward and reverse primers for the genes of interest, collagen type I and elastin, were designed with DNAMAN (Lynnon LLC. CA, USA). Primer parameters included product size between 100 to 300 base pairs (bp), primer length between 18 to 22 bases, melting temperature between 58 and 60 °C and GC content between 45 and 55 %. Resulting primers were excluded if primer sequence contained GGG or CCC sequences or if strong secondary or higher structures were predicted by Sigma Aldrich Design Tool Technical Data (https://www.sigmaaldrich.com/configurator/servlet/DesignTool). Gene accession numbers, primer sequences, annealing temperatures used and amplicon size are listed in Table 2.3. Peptidylprolyl isomerase A (Ppia) was used as the house keeping gene (289).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>Primer sequence</th>
<th>Annealing temperature (°C)</th>
<th>Amplicon size (bp)</th>
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<td>Ppia</td>
<td>BC083076.1</td>
<td>F: CAGATGATAAGGTCATCACGA R: GAATGGCTTCTATATCCCCAA</td>
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<td>217</td>
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<td>Collagen Type I</td>
<td>NM_007742.3</td>
<td>F: CCAGTGCGGTTATGACTT R: GCGGATGTTCTCAATCTGC</td>
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<tr>
<td>Elastin</td>
<td>NM_007925.3</td>
<td>F: GCTACTGCTTGGGAGAATG R: CTGCTGCTGTCGATTTCCTT</td>
<td>65</td>
<td>131</td>
</tr>
</tbody>
</table>

2.2.3.8.7 *Generation of double-stranded DNA (dsDNA) by endpoint polymerase chain reaction (PCR) for use in standard curve generation to calculate relative expression of genes*

Endpoint PCR was used to generate template DNA for production of a standard curve to allow for calculation of relative levels of gene expression in quantitative real time PCR (qPCR) (see below section 2.2.3.7.8). cDNA resulting from an mRNA sample of known, high concentration and low contamination was chosen and 20 ng of cDNA was mixed with the following components to final concentrations of 10 mM dNTP mix, 0.2 U/μL MangoTaq DNA polymerase, 1× MangoTaq colourless reaction buffer, 25 mM MgCl₂ solution, 250 nM forward primers, 250 nM reverse primers and made up to 20 μL with DNase/RNase free water. Samples were run using a CFX Connect Real-Time PCR Detection System (Bio-Rad, CA, USA) at 94 °C for 2 mins (polymerase activation and denaturation) followed by 36 cycles of: 94 °C for 5 secs (denaturation); 60 °C for collagen or 65 °C for Ppia and elastin (annealing); and 72 °C for 30 secs (extension). Samples were held at 4 °C before being run on a 3 % (w/v) agarose gel in 1× TBE buffer containing 0.05 % (v/v) SYBR Safe DNA gel stain at 90 V for 30 mins. 8 μL of dsDNA were run using the Hyperladder IV as a size reference.
Only pure dsDNA samples appearing at the correct size and as a single clear band, indicating no contamination, were used or stored at -20 °C.

2.2.3.8.8 qPCR of collagen type I and elastin

Quantification of mRNA levels was performed by qPCR using the CFX Connect Real-Time PCR Detection System with the SsoAdvanced Universal SYBR Green Supermix Kit (Bio-Rad, CA, USA) according to the manufacturer’s protocol. Samples were run in triplicate and the reaction (20 µL) consisted of 1× SYBR Green Supermix, 500 nM each for forward and reverse primers and 20 ng of cDNA. No Template Controls and No Enzyme Controls were run concurrently with samples to check for contamination. The reaction conditions were as follows: 95 °C for 3 mins (polymerase activation and denaturation); 40 cycles of: 95 °C for 10 secs (denaturation); 60 °C for collagen or 65 °C for Ppia and elastin for 15 secs (annealing); and 72 °C for 30 secs (extension). A melting curve analysis was performed at the end of the reaction to check for primer-dimer formation and contamination (Figure 2.8A and B). The absolute mRNA copy number of the genes of interest was calculated using a standard curve (Figure 2.8C) generated from dsDNA produced in section 2.2.3.7.7. Normalisation between samples was performed using Ppia.
Figure 2.9: (A) Melt curve showing no primer dimer formation, (B) melt curve with primer dimer formation and (C) standard curve generated from dsDNA for quantifying relative expression.

2.2.3.9 **One-step grafting of EDRT and IDRT in mice**

The dorsal area was shaved and cleaned with Betadine solution followed by sterile saline. Four, identical, 1 × 1 cm², full-thickness, skin excision wounds were surgically created adjacent to each other but separated by intact skin (Figure 2.9). Each wound was grafted with either EDRT+autograft, IDRT+autograft, an autograft or kept as an open wound. All autografts were full-thickness autografts and were sourced from neighbouring skin excisions in a clockwise pattern. Wounds grafted with EDRT/IDRT+autograft, grafting was performed by first suturing either EDRT or IDRT to the wound followed by immediate suturing of the graft over the substitute. Wounds were dressed with Atrauman and IV3000 for 7 days. Carprofen (5 mg/kg) was administered at the time of anaesthesia and on the first two days post-surgery for
analgesia. Wound contraction was monitored throughout the study with wound size measured on days 0, 3, 7, 14, 21 and 28 post-surgery using Visitrak (Smith & Nephew, NSW, Australia).

Mice were housed, monitored and given access to food and water as described in section 2.2.3.5. Mice were sacrificed using CO₂ on days 7, 14, 21 and 28 post-surgery and skin samples were collected for histological and IHC analysis.

2.2.3.10 Quantification of angiogenesis in one-step grafted EDRT and IDRT

Angiogenesis of wounds was examined at weeks 2 and 4 post-surgery by using the fluorescent probe, AngioSense 750 EX, with signal detection performed with the IVIS Lumina XR live imager (Thermal Fisher, Australia). AngioSense 750 EX is a near-
infrared labelled fluorescent macromolecule that works in vivo localising in blood vessels for 72 hrs. It allows imaging of vasculature leakage, such as areas undergoing angiogenesis, by pooling of the fluorescent probe in these areas resulting in a stronger signal. Each mouse was injected with 10 μL of the fluorescent probe diluted in sterile PBS to a concentration of 2 nmol/100 μL via a tail-vein injection. After 24 hrs the mouse was scanned using the IVIS live imager and the signal generated was captured for analysis of fluorescent radiant efficiency. The radiant efficiency was used to determine density of neo-vascularisation and angiogenesis.

2.2.3.11 Two-step grafting of EDRT and IDRT in mice

The first step of the procedure was to shave the dorsal area and clean with Betadine solution followed by sterile saline. Two, identical, 1 × 1 cm², full-thickness, skin excision wounds were surgically created adjacent to each other but separated by intact skin (Figure 2.10, step 1). Each wound was grafted with either EDRT or IDRT. All wounds were then individually covered with a silicone layer and dressed with Atrauman and IV3000 for 7 days. Carprofen (5 mg/kg) was administered at the time of anaesthesia and on the first two days post-surgery for analgesia. The second step of the procedure was 14 days later during which the silicone layer was removed and a full-thickness skin graft was cut from the dorsal area and grafted on the surface of an EDRT- or IDRT-regenerated wound bed (Figure 2.10, step 2). Autograft sites and grafted wounds were dressed with Atrauman and IV3000 for 7 days.
Mice were housed, monitored and given access to food and water as described in section 2.2.3.5. All mice were weighed on days 1-7, 9, 11 and 14 post-surgery and then after autografting on day 14 mice were weighed on days 15-21, 23, 25, 28 and 35 post-surgery.

### 2.2.4 Expression and statistical analysis of data

All data are expressed as mean ± standard error. Statistical significance was determined by analysis of variance with Bonferroni post-hoc analysis, independent t-tests or multiple linear regression analysis with least significant difference post-hoc.
analysis. Statistical significance was accepted at $p<0.05$ and significance is indicated in figures by * $p<0.05$, ** $p<0.01$ and *** $p<0.001$. 
Chapter 3

Development and characterisation of novel, electrospun, triple polymer scaffolds
Chapter 3  Development and characterisation of novel, electrospun, triple polymer scaffolds

3.1  Introduction

Electrospinning was first described over a century ago by Cooley and Morton in 1902 (290, 291) and regained attention in the 1990s due to a surging interest in the field of nanotechnology (292). The simplicity and low cost involved in the setup as well as the reproducibility and versatility of electrospinning make it an attractive form of scaffold fabrication (293). Particularly for skin tissue engineering, the nanoscale fibres are advantageous for several reasons: the high surface area is ideal for cell attachment and drug loading; the highly porous structure of the scaffold facilitates nutrient and waste exchange; the scaffolds have improved mechanical performance compared to traditional methods which is beneficial for the stresses placed on skin; and the fibrous scaffold structure closely resembles the structure of native ECM (293-295) (Figure 1.9).

3.1.1  Electrospinning setup and theory

The concept of electrospinning is similar to that of traditional spinning of fibres except an electric force is used to form the fibres rather than a mechanical force. The basic apparatus required is a voltage box, syringe pump, syringe/spinneret and collector (Figure 2.1). Electrospinning begins by dissolving polymers in a volatile, organic solvent and this polymer solution is loaded into a spinneret. A hemispherical drop of solution is held at the tip by surface tension and a high voltage power supply is used
to electrically charge the polymeric solution. The charge accumulates on the surface of the drop and as the charge increases the electric field causes the droplet to distort into a conical shape, commonly called the Taylor cone, and further increasing the charge causes the cone to diminish (253, 296) (Figure 2.1). At a critical voltage the repulsive charges within the solution overcome the surface tension of the solution and a jet is ejected from the tip of the cone (297).

A high viscosity solution is required to form a continuous jet as macromolecular entanglements prevent the solution from undergoing Rayleigh instabilities and breaking up into droplets, which would result in electrospraying rather than electrospinning (293, 296). Electric forces accelerate the jet away from the tip and the longitudinal stress caused by this stabilises the jet initially keeping it straight. At some point along its path the jet undergoes stress relaxation and as the jet approaches the oppositely charged or grounded collector the surface charge density on the jet increases (253, 298). When the charge is sufficiently high this, in conjunction with the stress relaxation, causes the jet to rapidly whip and small perturbations grow leading to large looping motions resulting in thinning of the jet (253, 293, 297). This is often called bending instability and causes accelerated solidification of the fluid jet to form dry fibres (297). As the solvent evaporates a continuous, nanoscale fibre is formed which accumulates on the collector forming a non-woven, fibrous, highly porous mat.

Electrospinning efficiency and physical properties of scaffolds such as fibre morphology and width, pore diameter, porosity, elasticity and strength are affected by electrospinning parameters and post-electrospinning modifications (Table 3.1). For example, increasing the polymer concentration can increase fibre diameter of electrospun gelatin (299), while surface morphology of polystyrene fibres can change from being smooth to pitted by increasing humidity to higher than 30 % (300). There
are also many different electrospinning setups such as the single syringe and flat collector setup used in this study which results in randomly aligned fibres or multiple spinnerets which can be used to obtain a combination of single-polymer fibres in the scaffold (301-303). If fibre alignment is desired a rotating drum/disc or parallel electrodes can be used (304-307) as seen with PLLA fibres collected on either a plate to give randomly oriented fibres (Figure 3.1A) or aligned PCL fibres collected on a rotating disc (Figure 3.1B). Another conformation is core-shell electrospinning which uses a coaxial, dual-capillary spinneret to encapsulate one component, which can be drugs or enzymes, inside another component (308, 309).

*Figure 3.1: Representative image of electrospun fibres randomly oriented from collection on a plate (A) and aligned fibres collected on a rotating disc (B).*
Table 3.1: Electrospinning parameters. Adapted from (310, 311).

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<thead>
<tr>
<th>Polymer parameters</th>
<th>Solution parameters</th>
<th>Process parameters</th>
</tr>
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<tbody>
<tr>
<td>• Molecular weight</td>
<td>• Viscosity</td>
<td>• Substrate properties</td>
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<tr>
<td>• Molecular weight distribution</td>
<td>• Viscoelasticity</td>
<td>• Solution flow rate</td>
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<tr>
<td>• Glass-transition temperature</td>
<td>• Concentration</td>
<td>• Distance between the needle tip and collector</td>
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<tr>
<td>• Solubility</td>
<td>• Surface tension</td>
<td>• Collector composition and geometry</td>
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<td></td>
<td>• Electrical conductivity</td>
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3.1.2 Electrospinning of polymers

Nearly 100 different polymers have been electrospun to date (294) including both synthetic and natural polymers as listed in section 1.7.2.1. Synthetic polymers are ideal for electrospinning as they are cost effective and have well-defined, predictable properties which translate into uniform behaviour during electrospinning (262). For tissue engineering, synthetic polymers permit fabrication of reproducible scaffolds, often have superior mechanical properties compared to natural polymers and are stable in aqueous environments without the need for cross-linking (262, 312). However, synthetic polymers can have poor cell-interactive properties, toxic degradation products and may be rejected after grafting (313).

Synthetic polymers can be functionalised by chemically modifying the polymer or by addition of signalling peptides or natural polymers to the electrospinning solution (314). Inclusion of biologically derived materials, i.e. natural polymers, provides biosignals
improving cell interactions such as attachment, migration, proliferation and differentiation (298, 315). For example, PCL was conjugated with heparin to electrospin nonwoven tubular scaffolds for use as a vascular substitute. These PCL-heparin fibres had improved hydrophilicity which could reduce inflammation and thrombus risk *in vivo* (316). However, there are difficulties involved in using these materials due to inter- and intramolecular interactions arising from chemical composition, 3D structures and polyelectric character of proteins (262). Many natural polymers also require post-electrospinning modifications in the form of cross-linking to maintain protein structure in aqueous environments (251).

### 3.1.3 Cellular Interactions with electrospun scaffolds

Electrospun scaffolds closely mimic ECM structure and as such are of great interest in tissue engineering. The scaffolds are highly porous and interconnected which facilitate exchange of cellular nutrients and metabolic waste (293) and also provide a more homogenous environment for cellular interaction compared to other fabrication techniques such as lyophilisation (278). Previous studies have shown a heterogeneous scaffold environment can have negative impacts on cell adhesion, morphology and ECM distribution (317, 318) which can lead to inferior biomechanical properties of deposited tissue (319).

The continuous, nanoscale fibres formed by electrospinning provide a large surface area which promotes cell adhesion (260-262). Electrospun fibres also promote cell proliferation compared to smooth films of the same materials (278). Cell orientation and morphology are also affected by electrospun fibres with cells aligning along fibres, which is especially evident when a scaffold has aligned fibres (254, 320). There have been a wide range of cells shown to have favourable interactions with electrospun
scaffolds including fibroblasts, keratinocytes, endothelial cells, smooth muscle cells, adipocytes and neural cells (278, 320-322).

3.1.4 Aim

This chapter aimed to utilise 2 natural polymers, collagen and elastin, and a synthetic polymer, PCL, to produce a scaffold which exhibited the advantageous properties of each polymer, namely, the promotion of cell interactions by collagen, imparting of elasticity by elastin and increased scaffold strength from PCL. The fabrication method of electrospinning was chosen as resulting scaffolds have a large surface area promoting cell adhesion and are highly porous which facilitates nutrient and waste exchange as well as cell infiltration. The triple-polymer scaffolds were characterised for morphological, mechanical and cell-interactive properties using HDFs and human epidermal keratinocytes.

3.2 Methods

Please refer to section 2.2.1 for all protocols used in this study.

3.3 Results and Discussion

The combination of collagen, elastin and PCL was tested in this study to determine if advantageous properties from all three polymers would be present in a single, composite, scaffold and glutaraldehyde, vapour cross-linking was used to stabilise the natural polymers and prevent immediate dissolution of the scaffold in an aqueous environment.
3.3.1 **Effect of electrospinning parameters on electrospinning efficiency**

3.3.1.1 **Effect of polymer composition on electrospinning efficiency**

A scaffold was produced using a polymer composition of 3C12E at a flow rate of 1 mL/h, an air gap of 15 cm and an applied electric potential of 20 kV. The scaffold showed a smooth surface before removal with minimal radiating fibres extending from the collector (Figure 3.2A). After removal, the scaffold had minimal wrinkling of the surface resulting from peeling the scaffold off the collector (Figure 3.2B). Fibre loss occurred when the scaffold was peeled from the collector which was evident by the white coat covering the surface of the brass collector (Figure 3.2C).
Figure 3.2: Electrospinning of various polymer solutions at a flow rate of 1 mL/h, air gap of 15 cm and applied electric potential of 20 kV. Images of the electrospun scaffolds produced using the 3C12E solution (A-C), 2.5C12E0.5P solution (D-F) and 2C12E1P solution (G-J) after electrospinning (left column), after removal from the collector (middle column) and fibre loss on the collector (right column).

A triple polymer scaffold was produced using 12 % (w/v) elastin, decreased collagen content of 2.5 % (w/v) and an addition of 0.5 % (w/v) PCL (2.5C12E0.5P). The resulting scaffold was soft and smooth after fabrication (Figure 3.2D) with easy removal. The
final, detached scaffold demonstrated more wrinkles on the surface of the scaffold and increased fibre loss was noticed compared to the 3C12E scaffold (Figure 3.2E). The increased fibre loss could be attributed to inclusion of PCL which may have decreased elasticity and flexibility of the fibres leading to fibres snapping and remaining on the collector. The final scaffold would still be considered suitable for cross-linking and further testing due to the even, low levels of fibre loss from removal (Figure 3.2F) and a resulting intact scaffold with uniform thickness.

Further reduction of the collagen content to 2 % (w/v) with an increase in PCL content to 1 % (w/v) (2C12E1P) resulted in poorer scaffold formation and droplet deformation (Figure 3.2G, orange arrows). Droplet deformation occurs when drops of solution are formed from the Taylor cone rather than a stable jet. This results in droplets contacting the surface of the scaffold, dissolving dry fibres and forming crater-like deformations on the scaffold surface. Under such situations, the electrospinning conditions are considered closer to electrospraying (323), hence the formation of droplets rather than jet. An unstable jet could be adjusted by changing the solution composition to increase the solution’s viscosity or by decreasing the applied electric potential to reduce the repulsive charge within the solution (296, 324-326). Droplet deformations also led to a poor scaffold with points of droplet deformation seen to be origins of fibre loss on the collector leading to increased fibre loss (Figure 3.2J). The resulting 2C12E1P scaffold was intact with similar amounts of wrinkling to that observed in the 2.5C12E0.5P (Figure 3.2H). However, due to fibre loss and droplet deformation this scaffold was not considered for further tests.

Surprisingly, a further decrease in collagen to 1.5 % (w/v) with increase of PCL to 1.5 % (w/v) (1.5C12E1.5P) improved scaffold fabrication (data not shown). No droplet
deformation and minimal fibre loss was observed compared to fabrication of 2.5C12E0.5P scaffolds.

This visual analysis of the effects of polymer composition on scaffold formation indicated that adding PCL to a CE solution had a variable effect on efficiency of scaffold formation and removal capability. Scaffold fabrication was possible with the CEP solutions and further optimisation could be performed with variations in flow rate and air gap.

### 3.3.1.2 Effect of flow rate on electrospinning efficiency

Electrospinning of the 1.5C12E1.5P solution at 1 mL/h, 15 cm and 20 kV, as previously discussed in section 3.3.1.1, resulted in a defect-free scaffold with minimal wrinkling and low, even fibre loss during removal. Electrospinning at an increased flow rate of 3 mL/h was abandoned due to solution dripping from the tip of the needle and therefore absence of a stable jet. An increase of the positive charge to the solution could overcome this issue as jet formation occurs when the repulsive force within the solution overcomes surface tension (292).

Decreased electrospinning efficiency was also observed with increasing flow rate during optimisation of electrospinning of the 3C12E solution. Electrospinning of the 3C12E solution at 1 mL/h, 15 cm and 20 kV resulted in a smooth scaffold with minimal wrinkling and fibre loss during removal (Figure 3.3A-C). Electrospinning at a flow rate of 3 mL/h resulted in a scaffold which appeared fluffier but the surface near the edge of the scaffold was thick and uneven with radiating fibres (Figure 3.3D). The final scaffold was quite wrinkled (Figure 3.3E) and had uneven fibre loss during removal from the collector (Figure 3.3F). The scaffold produced at 5 mL/h was poorer still with the entire surface of the scaffold appearing lumpy (Figure 3.3G) and, although the
scaffold was removed intact (Figure 3.3H), fibre loss during removal was uneven (Figure 3.3J). The lumpy surface could be a result of unstable Taylor cone formation and, therefore, a non-uniform flow rate. This unstable jet would result in varying amounts of fibre formation rather than a single, continuous jet and lead to uneven deposition on the collector.

Figure 3.3: Electrospinning at various flow rates using the 3C12E solution, an air gap of 15 cm and an applied electric potential of 20 kV. Images of the electrospun scaffolds produced using
1 mL/h (A-C), 3 mL/h (D-F) and 5 mL/h (G-J) after electrospinning (left column), after removal from the collector (middle column) and fibre loss on the collector (right column).

Conversely, electrospinning of 1.5C12E1.5P at 15 cm and 25 kV experienced improved electrospinning when flow rate was increased from 1 to 3 mL/h. When electrospun at 1 mL/h the scaffold exhibited droplet deformation but an increase to 3 mL/h resulted in a smooth scaffold with some fibre loss during removal. This improvement could be attributed to the formation of a continuous jet at a flow rate of 3 mL/h. As previously discussed, droplet deformation occurs when conditions are more akin to electrospraying. When the jet is stretched too thin, surface tension overcomes the repulsive charge within the solution and the jet breaks up forming droplets. Therefore, an increase in the flow rate had more macromolecular entanglements and a thicker, stable jet leading to the formation of fibres (296).

Increasing flow rate can improve scaffold formation by providing a sufficient amount of solution to eliminate droplet deformation, but in opposition higher flow rates can also provide excess solution resulting in solution dripping from the needle tip. The various outcomes are partially due to interaction with other electrospinning parameters. Primarily a high solution viscosity is needed for jet formation (296) and larger applied electric potentials with increasing flow rate are also necessary to provide the repulsive charge to form a stable Taylor cone and permit jet initiation (297).

### 3.3.1.3 Effect of air gap on electrospinning efficiency

Beginning with an air gap of 10 cm, the 1.512E1.5P solution was electrospun using a flow rate of 1 mL/h and an applied electric potential of 20 kV. The resulting scaffold was smooth with slight wrinkling and minimal fibre loss during removal. Fabrication with a larger air gap of 15 cm resulted in similar results to electrospinning at 10 cm,
but an air gap of 20 cm resulted in droplet deformation of the scaffold. Electrospinning with a 25 cm air gap was abandoned as solution was dripping from the needle tip. Electrospinning efficiency clearly decreased with increasing air gap at these parameters and this could result from the applied electric potential being insufficient at larger air gaps to support stable jet formation between the needle tip and collector. A previous study has observed that a larger applied electric potential increased the length of jet stability (323). Therefore, fabrication could be improved by increasing the applied electric potential while maintaining a constant polymer composition, flow rate and air gap. In addition, it was previously seen that insufficient repulsive charge at a high flow rate can also lead to solution dripping highlighting the interrelatedness between electrospinning parameters.

Conversely, increasing the air gap from 15 cm to 20 cm and 25 cm when electrospinning the 2C12E1P solution at 1 mL/h and 20 kV led to improved fabrication. The scaffold produced at 10 cm had uneven fibre loss originating from droplet deformation (Figure 3.4C). Electrospinning at 20 cm resulted in decreased, more even fibre loss during removal (Figure 3.4F) producing a scaffold with a more uniform environment and thickness. The scaffold fabricated at 25 cm was a further improvement from 20 cm. Fabrication at 25 cm resulted in good scaffold removal with almost little fibre loss observed on the collector (Figure 3.4J). Increasing the air gap most likely improved electrospinning by providing more time for solvent evaporation and collection of dry fibres. At the shorter air gaps of 15 and 20 cm insufficient solvent evaporation could have resulted in the deposition of droplets on the collector leading to fused fibres.
Figure 3.4: Electrospinning at various air gaps using the 2C12E1P solution, a flow rate of 1 mL/h and an applied electric potential of 20 kV. Images of the electrospun scaffolds produced using an air gap of 15 cm (A-C), 20 cm (D-F) and 25 cm (G-J) after electrospinning (left column), after removal from the collector (middle column) and fibre loss on the collector (right column).

Altering the air gap can either increase or decrease electrospinning efficiency, similar to the effects of flow rate. A larger air gap allows sufficient time for solvent evaporation and dry fibre formation but if the air gap is too large failed jet initiation can occur if the
applied electric potential is too weak. This can be addressed by increasing the applied electric potential; however, this can also lead to ionisation of the air resulting in decreased fibre deposition on the collector. Yet again the interplay of electrospinning parameters is evident.

3.3.1.4 **Effect of applied electric potential on electrospinning efficiency**

Scaffold production of the 1.5C12E1.5P solution was performed using a flow rate of 3 mL/h and an air gap of 15 cm. The initial applied electric potential of 20 kV was abandoned due to solution dripping from the tip. This was addressed by increasing the positive charge to the solution to 25 kV which increased the repulsive force within the solution and permitted jet formation. The resulting scaffold had some radiating fibres observed and removal from the collector had a low level of even fibre loss.

In comparison, electrospinning of the 1.5C12E1.5P solution at 1 mL/h, 15 cm and an initial applied electric potential of 20 kV experienced decreased electrospinning efficiency when the positive charge was increased to 25 kV. At the higher charge, droplet deformation was observed on the scaffold which in turn lead to poor scaffold removal. The inverse relationship between applied electric potential and flow rate is contrary to previous work (323) in which a positive correlation was observed between flow rate and applied electric potential. The differential effect of applied electric potential highlights the different electrospinning behaviours of polymer solutions due to variations in properties such as solution composition, viscosity, conductivity and surface tension.
3.3.1.5 *Interactions of electrospinning parameters electrospinning efficiency*

It is evident that electrospinning efficiency is dependent on numerous parameters and that parameters not only directly affect efficiency but also indirectly through interactions with other parameters. Formation of a scaffold with a smooth surface, even thickness and main fibre deposition on the surface has been seen to require not only fine-tuning of individual parameters, such as solution composition and air gap, but also changes of parameters in concert such as a decrease in flow rate for solutions with lower viscosity or larger applied electric potentials for larger air gaps. The four parameters examined in this study are only a small portion. There are many parameters such as solution viscosity, humidity and temperature which also affect scaffold formation and need to be examined (295, 298, 323, 327).

3.3.2 *Effect of inclusion of elastin on fibre morphology of non-cross-linked and cross-linked scaffolds*

Surface morphology of scaffolds was examined using SEM analysis and images showed a surface with ribbon-like fibres and no beads. Cross-sections of non-cross-linked, elastin-containing fibres were seen to be rectangular (Figure 3.5A and E) compared to CP fibres which appeared circular (Figure 3.5C). The rectangular morphology of elastin-containing fibres could be due to formation a thin polymer skin on the liquid jet during jet travel between the needle-tip and collector. This polymer skin collapses from atmospheric pressure when the solvent inside the polymer skin evaporates changing a circular cross section to elliptical and then a rectangular with the fibre taking on the appearance of a flat ribbon (328, 329). The ribbon-like morphology did not appear in CP fibres which could be explained by slower solvent evaporation preventing formation of a polymer skin.
Figure 3.5: SEM images of the surface of electrospun scaffolds showing cross-sections of fibres (yellow lines) before cross-linking (left column) and after cross-linking (right column). A representative CEP scaffold (A and B), CP scaffold (C and D) and CE scaffold (E and F). Magnification is 5000× and scale bars are 5 μm.

After crosslinking, no difference in fibre morphology was noticed with all fibres displaying a circular cross-section (Figure 3.5B, D and F). Additionally, elastin-
containing scaffolds displayed interconnected fibres which were not observed in CP scaffolds (Figure 3.5B and F). The change in fibre morphology of the CE and CEP scaffolds could be attributed to the higher content of natural polymer (elastin and collagen containing scaffolds) which would provide more sites for cross-linking leading to more interconnected fibres in scaffolds. Such interconnected networks of fibres are believed to facilitate cell infiltration by providing bridges for cell migration. In contrast, a CP scaffold was reported to delay cell migration and infiltration which could be attributed to the distinct fibres (330).

3.3.3 Effect of electrospinning parameters on pore diameter and fibre width of non-cross-linked, CEP scaffolds

Surface morphology of scaffolds was examined using SEM analysis and the pore diameters and fibre widths were quantified using ImageJ. Pore diameters were defined as the longest axis of the pore (Figure 2.2, blue lines) and fibre widths were measured at the cross-sections of individual fibres (Figure 2.2, yellow lines).

3.3.3.1 Effect of simultaneous increase in collagen content and decrease in PCL content on pore diameter and fibre width of non-cross-linked, CEP scaffolds

Stepwise increase of collagen content by 0.5 % (w/v) with corresponding decreases in PCL content resulted in estimated marginal means of pore diameter of 4.56 ± 0.26 μm for the 1.5C12E1.5P solution, 5.86 ± 0.35 μm for the 2C2E1P solution and 6.48 ± 0.41 μm for the 2.5C12E0.5P solution (Figure 3.6A). The mean pore diameters of the 2C12E1P and 2.5C12E0.5P solutions were shown to be significantly higher than the 1.5C12E1.5P solution (p<0.01 and p<0.001 respectively) indicating
the increase in collagen content and decrease in PCL content led to steady increases in pore diameter.

A similar trend was observed with fibre width increasing as collagen content increased and PCL content decreased (Figure 3.6B). The 1.5C12E1.5P solution had the lowest estimated marginal mean of 1.43 ± 0.03 μm, which increased to 1.6 ± 0.04 μm for the 2C12E1P solution and the 2.5C12E0.5P solution had the largest mean of 1.61 ± 0.05 μm. The 1.5C12E1.5P solution was shown to have significantly thinner fibre widths than both the 2C12E1P and 2.5C12E0.5P solutions (p<0.01). Previous electrospinning of pure, bovine, collagen type I has also demonstrated a positive, linear relationship between fibre width and collagen concentration (258).

Larger pores are favourable in a skin substitute as they will facilitate exchange of nutrients, waste and metabolites. In conjunction with the higher pore interconnectivity observed of CEP scaffolds compared to CP scaffolds, the large pores will also promote cell ingrowth of the scaffolds. The increasing fibre width observed, although shown to be significantly different between scaffolds, is not likely to have a noticeable effect on cell attachment. The CEP scaffolds had microscale fibres which have been shown to promote cell interactions (260). The concurrent increase in both pore diameter and fibre width is also expected as previous studies of statistical modelling and experimental data have shown this relationship (331-337).
Figure 3.6: Graphical representation of estimated marginal means of pore diameter (A) and fibre width (B) plotted against simultaneous increases in collagen and decreases in PCL content for all, non-cross-linked, CEP scaffolds tested (**p<0.01 and ***p<0.001).
Pore diameter and fibre width were found to increase with increasing flow rate. At the lower flow rate tested, 1 mL/h, the estimated marginal mean of pore diameters was 4.62 ± 0.31 μm which increased to 6.65 ± 0.33 μm at 3 mL/h (Figure 3.7A). This increase in pore diameter was found to be significant (p<0.001). The change in fibre width was also significant with an increase from 1.28 ± 0.04 μm at 1 mL/h to 1.81 ± 0.04 μm at 3 mL/h (p<0.001) (Figure 3.7B). The effect of increasing flow rate leading to increased fibre width has been observed in previous studies (332, 338). As discussed above, the larger pores and microscale fibres will have advantageous effects on cell interactions such as attachment, infiltration (260) and growth by improving exchange of fluids.
Figure 3.7: Graphical representation of estimated marginal means of pore diameter (A) and fibre width (B) plotted against flow rate for all, non-cross-linked, CEP scaffolds tested (**p<0.001).
3.3.3.3 **Effect of air gap on pore diameter and fibre width of non-cross-linked, CEP scaffolds**

Increasing the air gap resulted in decreasing estimated marginal mean of pore diameter of 5.96 ± 0.64 μm at 10 cm, 5.78 ± 0.30 μm at 15 cm, 5.51 ± 0.23 μm at 20 cm and 5.28 ± 0.45 μm at 25 cm. These differences were shown to be insignificant (Figure 3.8A). Decreased pore diameter was expected since the jet had a longer path to travel undergoing more instabilities and elongation leading to fibre thinning. The positive relationship between pore diameter and fibre width would then dictate smaller pores would result as air gap increased.

However, fibre width was shown to increase from 1.41 ± 0.08 μm at 10 cm to 1.66 ± 0.03 μm and then to 1.71 ± 0.03 μm at 20 cm before decreasing back to 1.41 ± 0.06 μm at 25 cm (Figure 3.8B). Significance was observed between 10 and 15 cm (p<0.01), 10 and 20 cm, 15 and 25 cm as well as 20 and 25 cm (p<0.001). The cause of this surprising result is unclear, especially since a positive relationship appeared between pore diameter and fibre width with collagen/PCL content and flow rate.
Figure 3.8: Graphical representation of estimated marginal means of pore diameter (A) and fibre width (B) plotted against air gap for all, non-cross-linked, CEP scaffolds tested (** p<0.01 and ***p<0.001).
3.3.3.4 Effect of applied electric potential on pore diameter and fibre width of non-cross-linked, CEP scaffolds

The applied electric potential had a variable effect on both pore diameter and fibre width. Estimated marginal mean of the pore diameter decreased from 5.22 ± 0.28 µm at a potential of 20 kV to 4.05 ± 0.41 µm at 25 kV; however, increasing the potential to 30 kV resulted in a larger pore diameter of 7.63 ± 0.47 µm (Figure 3.9A). Conversely, fibre width mean increased from 1.37 ± 0.03 µm at 20 kV to 1.71 ± 0.05 µm at 25 kV and then decreased to 1.57 ± 0.06 kV at a potential of 30 kV (Figure 3.9B). As seen with air gap, there was no relationship observed between pore diameter and fibre width.

The two electrospinning parameters of increasing collagen content/decreasing PCL content as well as increasing flow rate were shown to form larger pores while maintaining microscale fibres. Air gap and applied electric potential were seen to have varying effects on both pore diameter and fibre width. This lack of relationship observed between pore diameter and fibre width was unexpected as previous studies have shown a positive relationship between the two and further investigation of these two parameters would be useful to elucidate if any relationship exists with pore diameter and fibre width (331-337). Despite this, increasing natural polymer content while decreasing synthetic polymer content can be used to improve scaffold structure by not only increasing pore diameter but by also improving fibre and pore interconnectivity to facilitate cell infiltration and fluid exchange.
Figure 3.9: Graphical representation of estimated marginal means of pore diameter (A) and fibre width (B) plotted against applied electric potential for all, non-cross-linked, CEP scaffolds tested (** p<0.01 and ***p<0.001).
3.3.4 Effect of cross-linking on pore diameter and fibre width of CEP scaffolds

Six scaffolds were chosen for cross-linking based on favourable morphological properties of large pores and microscale fibres. Four CEP scaffolds were chosen, one CP and one CE scaffold. Scaffold designations and electrospinning parameters are listed in Table 2.2.

Despite the intermittent trends observed between morphological properties and individual electrospinning parameters the effect of cross-linking on scaffolds was variable with no significance observed (Figure 3.10).

3.3.4.1 Mechanical properties CEP scaffolds

Tensile tests were used to examine physical properties of electrospun scaffolds. The CP scaffold was shown to have a significantly higher tensile modulus of 1411.8 ± 25.0 kPa compared to 108.0 kPa in CE, 108.0 ± 18.2 kPa in CEP 1, 53.0 ± 4.9 kPa in CEP 2, 66.0 ± 8.7 kPa in CEP 3 and 153.7 ± 9.0 kPa in CEP 4 (p<0.001; Figure 3.11A). The lower tensile moduli observed in scaffolds fabricated with elastin confirms the presence of elastin increases elasticity (339, 340). Energy loss, an indicator of good shape retention at low values, was also calculated and no trend was observed with 36.7 ± 1.5 % in CP, 82.0 % in CE, 73.0 ± 0.0 % in CEP 1, 24.0 ± 1.2 % in CEP 2, 18.7 ± 0.3 % in CEP 3 and 69.0 ± 0.0 % in CEP 4 (Figure 3.11B).

CEP 1 and CEP 2 were chosen for further characterisation based on improved elasticity and favourable morphological properties.
Figure 3.10: Changes in average pore diameter (A) and fibre width (B) of cross-linked, electrospun scaffolds before and after cross-linking.
Figure 3.11: Graphical representation of the tensile moduli (A) and energy loss (B) of electrospun scaffolds (***, p<0.001).
3.3.5 **Pore diameter, fibre width and porosity of CEP 1 and CEP 2**

CEP 1 and CEP 2 were chosen for further cell characterisation due to the favourable morphological and mechanical properties demonstrated by both scaffolds. CEP 1 had an average pore diameter of $5.30 \pm 0.38 \mu m$ which was significantly smaller than the average pore diameter of CEP 2 at $7.83 \pm 0.42 \mu m$ ($p<0.001$; Figure 3.12). The pores of CEP 1 and CEP 2 should be sufficiently large to allow cell infiltration as a previous study using electrospun, CP scaffolds demonstrated HDF infiltration with pore diameters approximately $5 \mu m$ in diameter (277). In contrast, average fibre width of CEP 1 was significantly larger than CEP 2 at $1.69 \pm 0.08 \mu m$ and $1.41 \pm 0.07 \mu m$ respectively ($p<0.01$; Figure 3.13) which may affect cell-seeding efficiency as thinner fibres results in less surface area for cell attachment. Differences between CEP 1 and CEP 2 could not be predicted or explained due to the numerous differences in electrospinning parameters during fabrication; however, it was surprising pore diameter and fibre width did not positively correlate since previous statistical modelling and experimental data supported this relationship (331-337). CEP 3 was not chosen due to poor removal from the scaffold with uneven fibre (Figure 3.14) loss which would affect scaffold uniformity and integrity and CEP 4 was not chosen due to high amounts of fibre loss during removal (Figure 3.2D-F).
Figure 3.12: Average pore diameters of CEP 1 and CEP 2 (** p<0.01).

Figure 3.13: Average fibre widths of CEP 1 and CEP 2 (** p<0.01).
Figure 3.14: Electrospinning of 2.5C12E0.5P solution at a flow rate of 1mL/h, an air gap of 20 cm and an applied electric potential of 25 kV after electrospinning (left image), after removal from the collector (middle image) and fibre loss on the collector (right image).

Sections of cross-linked CEP 1 and CEP 2 were stained using H&E to visualise the fibres. Images of the stained cross-sections were converted to binary images and both scaffolds were seen to have fibrous structures (Figure 3.15). Based on the significant differences in pore diameter and fibre width between CEP 1 and CEP 2, it was surprising that porosity measurements showed no significant difference between the scaffolds with 74.0 % for CEP 1 and 70.4 % for CEP 2 (Figure 3.16). This lack of significance could simply be due to variation within and between scaffolds produced as Figures 3.11 and 3.12 show a range of pore diameters and fibres widths for both scaffolds.
Figure 3.15: H&E images (A and B) of CEP 1 (left column) and CEP 2 (right column) converted to binary images (C and D) for quantification of porosity. Magnification is 20× and scale bars are 200 μm.
3.3.6 **HaCaT behaviour on CEP 1 and CEP 2**

HaCaTs were seeded onto CEP 1 and CEP 2 to investigate keratinocyte interaction with scaffolds examining cell attachment, migration and proliferation. The morphology of HaCaTs on the surface of electrospun scaffolds was examined on days 1 and 14. SEM images showed flattened and attached HaCaTs on both scaffolds (Figure 3.17A and B, yellow arrows), indicating differences in pore diameter and fibre width had no effect on morphology of HaCaTs attached to the scaffolds. Migration of HaCaTs was observed on CEP 1 (Figure 3.17A, orange arrows) and the beginnings of a subconfluent cell layer forming on CEP 2 (Figure 3.17B, outlined in green). Cell layers were observed on both scaffolds on day 14 suggesting favourable biocompatibility of the electrospun scaffolds (Figure 3.14C and D). H&E staining confirmed the presence of a confluent cell layer on day 28 (Figure 3.18C and D) while also revealing HaCaTs
formed a subconfluent layer on day 1 (Figure 3.18A and B). Cells remained on the surface of the scaffolds with no cell infiltration over 28 days.

Figure 3.17: SEM images of flattened and attached HaCaTs (yellow arrows), migrating HaCaTs (orange arrows) and a subconfluent cell layer (outlined in green) on the top surface of HaCaT-seeded CEP 1 (left column) and CEP 2 (right column) at day 1 (A and B) and day 14 (C and D) post-seeding. Magnification is 1000× and scale bars are 10 μm.
HaCaTs attached and remained on the surface of electrospun scaffolds. These are desirable behaviours since keratinocytes are responsible for regenerating the epidermal layer of skin. The formation of subconfluent layers of cells on day 1 would facilitate keratinocyte growth on the scaffolds through the expression of autocrine factors such as TGF-α, amphiregulin and epiregulin (341). Accelerated cell growth of keratinocytes could lead to more rapid development of an epithelial layer that would accelerate wound healing by promoting re-epithelialisation. DAPI staining of nucleic material and fluorescence analysis displayed an intact layer of HaCaTs on the surface of the scaffolds (Figure 3.19). Detachment of HaCaT cells were noticed on CEP 2 at
day 28 which may have been caused by physical effects during tissue processing (Figure 3.19F).

Figure 3.19: DAPI images of HaCaT-seeded CEP 1 (left column) and CEP 2 (right column) at day 1 (A and B), day 14 (C and D) and day 28 (E and F) post-seeding. Magnification is 10× and scale bars are 400 μm.
In an attempt to circumvent the cell layer separation issue arising from processing for paraffin embedding an alternative method of cryosectioning of the seeded samples was tested. The samples were fixed in 4 % (v/v) paraformaldehyde for 15 mins at RT, then embedded in OCT compound and finally frozen in 100 % ethanol chilled with dry ice. Section were cut by cryosectioning with thicknesses varying from 5 μm to 10 μm; however, all samples showed visible separation of the cell layers from the scaffold during sectioning and this technique was abandoned for analysis of in vitro cell localisation.

Cell numbers of HaCaTs on CEP 1 and CEP 2 were measured using a haemocytometer on day 1 which revealed both electrospun scaffolds had good cell-attachment with 98.2 ± 0.4 % on CEP 1 and 96.4 ± 0.6 % on CEP 2 (Figure 3.20). Furthermore, CEP 1 demonstrated a significantly higher cell attachment compared to CEP 2 (p<0.05) which could be due to the smaller pores of CEP 1 (5.30 ± 2.09 μm) compared to CEP 2 (7.83 ± 0.42 μm) (P<0.001). The higher cell attachment may also be explained by the significantly larger fibres of CEP 1 (1.69± 0.08 μm) compared to CEP 2 (1.41 ± 0.07 μm) (p<0.01) and the smaller average pore diameter of CEP 1 may have facilitated cells bridging the pores. The microscale pores of the scaffolds may have aided HaCaT migration and confluency as a previous study has shown that intercellular adhesion between keratinocytes is sufficiently strong to pull neighbouring cells along over non-adhesive substrates (342).
HaCaT proliferation on both CEP 1 and CEP 2 scaffolds was also assessed (Figure 3.21). CEP 1 was shown to have significantly higher HaCaT numbers compared to CEP 2 over 28 days (Figure 3.21). However, despite the higher number of HaCaTs on CEP 1, CEP 2 displayed a consistent cell proliferation over 28 days with 10,955 ± 1,665 cells on day 7, 18,708 ± 7,888 cells on day 14, 47,896 ± 7,420 cells on day 21 and extensive cell proliferation of 221,608 ± 29,729 cells. Comparatively, CEP 1 showed a slight drop in cell number from 43,501 ± 10,259 cells on day 7 to 20,248 ± 9,334 cells on day 14, but thereafter cell number increased to 72,332 ± 6,491 cells on day 21 and finally 240,229 ± 21,635 cells on day 28.
Figure 3.21: HaCaT proliferation over 28 days on CEP 1 and CEP 2 (* p<0.05, ** p<0.01, *** p<0.001).

3.3.7 HDF behaviour on CEP 1 and CEP 2

HDF infiltration and proliferation on CEP 1 and CEP 2 were investigated on days 1 and 14 post-seeding. SEM analysis showed HDF migration on days 1 and 14 on the surface of CEP 1 and CEP 2 (Figure 3.22, yellow arrows). These HDF interactions with electrospun scaffolds indicate favourable scaffold environments. Additionally, H&E images showed initial HDF infiltration on day 14 (Figure 3.23A and B, black arrows) and both H&E and DAPI images showed complete infiltration of both scaffolds by day 28 (Figure 3.23C and D and Figure 3.24C and D respectively). This is in line with previous studies which have shown cell infiltration into scaffolds with 5 μm pores (277) despite the previously established pore range of 20-120 μm required for cell infiltration (343). No confluent cell layers of HDFs were observed on the surface of the
scaffold compared to HaCaT cells, suggesting both electrospun, CEP scaffolds had favourable internal structure to support HDF infiltration. This indicates both scaffolds could be used to produce a living, skin substitute since scaffolds supported HDF infiltration and HaCaT localisation and confluency on the scaffold surface.

Figure 3.22: SEM images of migrating HDFs (yellow arrows) on the top surface of HDF-seeded CEP 1 (left column) and CEP 2 (right column) at day 1 (A and B) and day 14 (C and D) post-seeding. Magnification is 1000× and scale bars are 10 μm.
Figure 3.23: H&E images of infiltrating HDFs (black arrows) on HDF-seeded CEP 1 (left column) and CEP 2 (right column) at day 14 (A and B) and day 28 (C and D) post-seeding. Magnification is 20× and scale bars are 200 μm.
Figure 3.24: DAPI images of HDF-seeded CEP 1 (left column) and CEP 2 (right column) at day 1 (A and B) and day 28 (C and D) post-seeding. Magnification is 10× and scale bars are 400 μm.

Previous work with an electrospun, CP scaffold showed no cell infiltration with pore diameters in the range of 1.0-13.6 μm (330). The pore diameters of the present CEP scaffolds are similar to those of the CP scaffold. Therefore, the HDF infiltration observed on CEP scaffolds could be attributed to the elastin in the scaffolds. It has been seen the repeating, Val-Gly-Val-Ala-Pro-Gly peptide in elastin is chemotactic for fibroblasts and as such the inclusion of elastin during fabrication of the scaffolds resulted in fibres with biosignals for the migration of fibroblasts (344). This could be confirmed by observing HDF infiltration in different CEP scaffolds with varying amounts of elastin. A previous study has also shown a pure elastin scaffold supported
cell infiltration with subsequent incorporation of collagen leading to enhanced infiltration (273).

HDF infiltration was also seen to modify the structure of CEP 1 and CEP 2 with fibre distribution appearing altered on day 28 compared to the original structure of the scaffolds on day 1 (Figure 3.24). Electrospun scaffolds seeded with HDFs lost their original shape with non-uniform thickness and varying fibre distribution. The change of scaffold structure was more apparent in images of CEP 1 which did not appear as a strip of scaffold on day 28 and CEP 2 had large pores and uneven thickness. These results could indicate scaffold breakdown and further investigation for the presence of enzymes, such as MMPs, would elucidate the cause of scaffold degradation.

Assessment of HDF numbers on day 1 revealed CEP 1 and CEP 2 had similar cell seeding efficiency of 92.8 ± 1.5 % and 92.4 ± 1.4 %, respectively (Figure 3.25). Cell proliferation rate showed cell numbers on day 7 were also similar between both scaffolds with 20,976 ± 6,101 cells on CEP 1 and 11,968 ± 6,901 cells on CEP 2 (Figure 3.26). On day 14, CEP 1 had significantly higher cell numbers with 54,389 ± 5,549 cells compared to 26,715 ± 711 cells on CEP 2 (p<0.001). It was also found that CEP 1 had significantly higher numbers of HDFs on day 21 of 27,781 ± 16.043 cells compared to 153,984 ± 34,064 cells on CEP 2. This result, combined with previous data showing CEP 1 had higher HaCaT proliferation on the surface, suggested that CEP 1 is more suitable as a skin substitute. A drop in HDF numbers was observed on day 28 to 141,921 ± 32,720 cells on CEP 1 and 142,247 ± 41,657 cells on CEP 2.
Figure 3.25: HDF-seeding efficiency on CEP 1 and CEP 2.

Figure 3.26: HDF proliferation over 28 days on CEP 1 and CEP 2 (* p<0.05, ** p<0.01).
The increase in cell number from days 7 to 21 support the findings from the histological analysis that the electrospun scaffolds provide a favourable environment for HDFs. Additionally, the significantly higher number of HDFs on CEP 1 at days 14 and 21 suggest that the higher collagen content and/or lower PCL content are conducive to HDF-scaffold interactions. The unexpected drop in HDFs from day 21 to 28 on both scaffolds could result from limited mass transport and nutrition supply to the internal area of the scaffold which causes cell death. The reduction of cell number may also be explained by the structural change of the scaffold as noticed in the DAPI images (Figure 3.24) which leads to closure of pores and a sealed structure inside the scaffolds and subsequent cell death.

3.3.8 Co-culture of HaCaTs and HDFs on CEP 1 and CEP 2

HDFs were seeded onto CEP 1 or CEP 2 for one week before seeding of HaCaTs. Co-culture of skin cells was analysed for cell-scaffold interactions on days 1, 14 and 28 post-seeding. SEM analysis revealed a confluent layer on day 1 (Figure 3.27A and B); however, the two cell types were indistinguishable. The confluent cell layer was maintained on days 14 and 28 with the surface appearing rough (Figure 3.27C-F).
Figure 3.27: SEM images of the top surface of HaCaT- and HDF-seeded CEP 1 (left column) and CEP 2 (right column) at day 1 (A and B), day 14 (C and D) and day 28 (E and F) post-seeding. Magnification is 1000× and scale bars are 10 μm.
Fluorescence analysis showed multilayers and aggregation of cells detached from CEP 1 and CEP 2 on day 1 (Figure 3.28A and B). This cell detachment is likely due to sample processing since a cell layer was observed attached to the scaffold in the SEM images. Partial separation of the HaCaT layer from CEP 2 was also previously noticed in DAPI images (Figure 3.19F). Cell layers were absent on days 14 and 28 in DAPI images (Figure 3.28C-F). Fluorescence analysis also revealed minimal cell infiltration over the 28 days which was unexpected since HDF infiltration was previously observed in the HDF-only study, although there appears to be some nuclei fragments in the day 28 CEP 2 sample (Figure 3.28F). Moreover, accelerated scaffold expansion and breakdown was observed compared to HDF-seeded scaffolds. This breakdown of the scaffolds in vitro could be due to the inhibition of ECM deposition as basic fibroblast growth factor, secreted by keratinocytes, could inhibit TGF-β1 which stimulates production of elastin and collagen type I (345, 346). Rapid scaffold degradation is undesirable as it may not provide sufficient mechanical support for cell infiltration and tissue regeneration in wound healing. Further modifications such as the use of a coaxial electrospinning setup with PCL in one solution and a CE blend in another could allow for more durable scaffolds that still provide sufficient biosignals. The natural polymer fibres would promote cell attachment and migration and as the natural fibres are degraded or subjected to contraction, the PCL-only fibres would provide an alternate, more resilient, fibrous network for cell attachment while scaffold remodelling occurred.
Figure 3.28: DAPI images of HaCaT- and HDF-seeded CEP 1 (left column) and CEP 2 (right column) at day 1 (A and B), day 14 (C and D) and day 28 (E and F) post-seeding. Magnification is 10× and scale bars are 400 μm.
3.4 Summary

Electrospun, CEP scaffolds were successfully produced with modification of pore diameter, porosity and fibre width. Alteration in flow rate, air gap and applied electric potential were studied aiming to improve scaffold fabrication. It was found increasing natural polymer content and higher flow rates increased pore diameters which could result in facilitated cell infiltration and remodelling. Tensile testing of the scaffolds revealed the inclusion of elastin produced more elastic scaffolds. CEP 1 and CEP 2 were chosen based on advantageous morphological and enhanced mechanical properties. Investigations with skin cell cultures revealed both scaffolds supported formation of a confluent layer of HaCaT cells and full infiltration of HDFs over 28 days; however, co-cultures were inconclusive due to processing issues. Both CEP 1 and CEP 2 displayed promising cell-scaffold interactions supporting normal cell behaviour; however, CEP 1 had higher HaCaT proliferation at all time points and higher proliferation of HDFs at some time points. There was also no observable scaffold degradation observed by day 28 in either scaffold indicating there was no scaffold remodelling biodegradation occurring in vitro. This could be attributed to increased protein stability of the scaffold from cross-linking. The two scaffolds are good candidates for further testing using animal models to examine biocompatibility.
Chapter 4

Morphological characterisation and cell-interactive properties of novel elastin-Integra Dermal Regeneration Template
Chapter 4  Morphological characterisation and cell-
interactive properties of novel elastin-Integra
Dermal Regeneration Template

4.1  Introduction

Burn injuries are some of the most traumatic and debilitating injuries, but with improved intensive care and advances in surgical techniques patients can survive burns affecting over 90% TBSA (183). The gold-standard treatment for burn injuries are autologous skin grafts harvested from healthy, undamaged sites. However, patients with severe burn injuries affecting over 50% TBSA lack donor sites. Skin substitutes have been developed to address this shortage, but patients can still suffer from a loss in skin functionality, especially elasticity and flexibility.

4.1.1  IDRT

IDRT is an acellular, bilaminar, dermal substitute first introduced in 1977 (347). The substitute is a lyophilised scaffold cross-linked with glutaraldehyde (82) consisting of a matrix of bovine, collagen type I and a shark GAG, chondroitin 6-sulphate, in a ratio of 92:8 with a temporary, artificial, epidermal layer of silicone bonded to the collagen matrix (348). Once the matrix is vascularised the silicone layer can be removed and covered with a thin split-thickness autograft to form the epidermal layer (349). In 2010 a single-layer IDRT was released consisting of only the collagen-GAG matrix. Single-layer IDRT was designed for use in conjunction with IDRT to fill deeper wounds, but has since been used in one-step grafting procedures (349, 350).
IDRT was intended for use on burn survivors of partial- to full-thickness, extensive, life-threatening injuries who had insufficient skin donor sites for autografting (348). The immediate issues of massive infection and severe fluid loss were taken into consideration during its design while also addressing the long-term issues of disfiguring scars and crippling contractures (84). In the years since, IDRT has been used not only for burn survivors but in a wide array of circumstances in which skin substitutes are needed such as deglovement injuries (351), scar contracture release procedures (352), excision of giant nevi (353) and skin avulsion injuries (350). IDRT allows for wound bed regeneration and its effectiveness has been shown by its higher elasticity compared to split-thickness skin grafts in vivo (354). It has also been shown to result in a thicker dermis compared to MatriDerm, a non-cross-linked matrix of collagens type I, II and V coated with α-elastin hydrolysate from bovine ligamentum nuchae, in animal models (355).

However, newly produced skin is not always morphologically or functionally normal (73, 101) with new skin having reduced elastic function in comparison to normal skin in the long-term (356). A study revealed IDRT induced more foreign body reaction than Alloderm (LifeCell, Branchburg, NJ, USA) due to macrophages clearing the cross-linked IDRT in a mouse model (357). Another study compared IDRT to an artificial, dermal substitute called Hyalomatrix (Anika, Therapeutics Bedford, MA, USA), which has a dermal layer of hyaluronic acid derivative and an epidermal layer of semipermeable silicone. It was found that Hyalomatrix had better cell regulation and stimulatory activity resulting in better deposition of ECM (354). Clearly, there are still aspects of IDRT that can be improved, especially in regards to cell-scaffold interactions. The presence of elastin in the scaffold could achieve this by not only
increasing elasticity of the substitute but also by improving fibroblast infiltration and thereby wound healing.

4.1.2 Recombinant, human tropoelastin

Elastin is a major component of elastic fibres and is formed through the cross-linking of its precursor tropoelastin, a 60-70 kDa protein depending on splice variants (176). Tropoelastin is encoded by separate exons and characterised by alternating hydrophobic and hydrophilic domains (178). The hydrophobic domains are involved in monomer association in solution and also elastic function of elastin while the hydrophilic domains are implicated in polymerisation of the monomer through cross-linking (179).

Elastin is a durable protein with a half-life of approximately 70 years (181) and is mainly formed during late fetal and early neonatal periods (183). It is synthesised from several cell types including fibroblasts (181). Elastin is a key structural and mechanical component in the elastic-fibre network which gives skin its resilience, texture and quality while also possessing cell signalling properties which promote cell responses such as cell attachment, proliferation and differentiation (358-362). Previous studies have shown scaffolds electrospun from tropoelastin have similar stiffness to native elastin and that these electrospun scaffolds support fibroblast infiltration, fibroblast persistence and scaffold remodelling in vitro (338). Further studies have shown that these electrospun scaffolds also enhanced fibroblast proliferation and migration in vitro compared to IDRT and in vivo the electrospun, tropoelastin scaffolds also supported fibroblast infiltration, collagen deposition and angiogenesis (273).
4.1.3 **Aims**

The well-established use of IDRT in a clinical setting has elucidated properties that can be improved. It is with this knowledge the elastin-modified version of IDRT, called EDRT, has been developed by incorporating 10 % (w/w) recombinant, human tropoelastin, purified from *Escherichia coli* as previously described (363, 364), during fabrication. The aim of this chapter was to characterise the morphological, mechanical and cell-interactive changes from the presence of elastin in the substitute.

4.2 **Methods**

Please refer to section 2.2.2 for all protocols used in this study.

4.3 **Results and Discussion**

4.3.1 **Pore diameter and porosity of EDRT and IDRT**

EDRT and IDRT displayed soft, hydrogel characteristics (Figure 4.1). SEM analysis of EDRT and IDRT revealed both scaffolds had a network of fibrous material resembling a sponge (Figures 4.2). The pores of both scaffolds were irregular in both shape and size as well as being interconnected. Quantification of the range of pore diameters revealed IDRT had average pore diameters of $272.5 \pm 65.0 \mu m$. Comparatively, inclusion of 10 % tropoelastin during fabrication significantly increased average pore size to $375.0 \pm 48.4 \mu m$ in EDRT ($p<0.05$; Figure 4.3).
Figure 4.1: Photographs of EDRT (A) and IDRT (B) sections.

Figure 4.2: SEM images of the surface of acellular EDRT (A) and IDRT (B) showing pore diameter (blue lines). Magnification is 100× and scale bars are 100 μm.
Porosity was visualised by converting H&E stained cross-sections of EDRT and IDRT to binary images (Figures 4.4). The porosity of EDRT was shown to be significantly higher at 88.3 ± 2.4 % compared to IDRT at 83.8 ± 2.7 % (p<0.01; Figure 4.5) which is in accordance with the significantly larger macropores observed in EDRT. The larger pores and subsequently higher porosity observed in EDRT could be attributed to the suspected increased elasticity, imparted by the presence of elastin, resulting in a more flexible fibrous network. Mechanical tests will be conducted to examine scaffold elasticity and rigidity. H&E analysis confirmed SEM results that elastin had no effect on IDRT structure with both scaffolds displaying a sponge-like appearance (Figure 4.2).
Figure 4.4: H&E images (A and B) of EDRT (left column) and IDRT (right column) converted to binary images (right column) for quantification of porosity.
Figure 4.5: Porosity of EDRT and IDRT calculated from binary images (** p<0.01).

4.3.2 Mechanical properties of EDRT and IDRT

Mechanical properties of the scaffolds were investigated using compression tests. IDRT had a compressive Young’s modulus of 3.1 ± 0.9 kPa which was significantly higher than EDRT’s at 1.1 ± 0.3 kPa (p<0.001) (Figure 4.7). Revealing that the addition of 10 % (w/w) tropoelastin increased rigidity of IDRT. This is in line with a previous study which found an electrospun, CE scaffold was stiffer than an elastin-only scaffold (365). From a mechanical perspective, a lower stiffness indicates that EDRT deforms more than IDRT under the same loading. From a materials perspective, it suggested that the EDRT scaffolds has a lower degree of cross-linking and a higher swelling ratio, which would translate into less resistance to compression and the reduced hysteresis (Figure 4.6).
Figure 4.6: Compressive stress-strain curve of EDRT and IDRT scaffolds (366).

Figure 4.7: Graphical representation of the compressive Young’s modulus, calculated in the linear region of 0.05-0.1 mm/mm from Figure 4.6 of EDRT and IDRT scaffolds (*** p<0.001) (366).
Figure 4.8: Graphical representation of the energy loss in 0.25 mm/mm strain level from Figure 4.6 of EDRT and IDRT scaffolds (** p<0.01) (366).

The ultimate compressive strain results revealed that EDRT had significantly higher elasticity of 0.37 ± 0.02 % compared to IDRT 0.26 ± 0.02 % (p<0.01; Figure 4.9) which again translates into a better skin substitute as increased flexibility and elasticity of the scaffold allows higher deformability. The confirmation of increased elasticity of EDRT could account for the larger pores observed in EDRT providing more flexibility and deformability to relieve stress within the network.
Figure 4.9: Graphical representation of the ultimate compressive strain of EDRT and IDRT scaffolds (** p<0.01) (366).

4.3.3 HaCaT behaviour on EDRT and IDRT

Cell-seeding efficiency was examined at 24 hrs. The results showed both scaffolds had a high level of HaCaT attachment with seeding efficiency over 92 %, but IDRT was shown to have a slightly higher efficiency with 96.9 ± 0.2 % compared to EDRT, 92.6 ± 0.6 % (p<0.001; Figure 4.10) which could be due to the smaller average pore size of IDRT resulting in more cells being captured in the scaffold.

HaCaT proliferation was assessed over 28 days and cell numbers gradually increased on scaffolds with 131,773 ± 4,531 cells on EDRT on day 7 to 148,488 ± 16,729 cells on day 14 followed by a large increase to 486,940 ± 36,047 cells on day 21 and 584,483 ± 17,277 cells on day 28 (Figure 4.11). Likewise, on IDRT had 136,200 ± 9,029 cells on day 7 with an increase to 153,129 ± 6,702 cells on day 14
and large increases on day 21 to 407,590 ± 15,529 cells and 560,837 ± 56,292 cells on day 28 (Figure 4.11). There were no significant differences observed in cell numbers between EDRT and IDRT at all-time points or between time points.

Figure 4.10: Cell-seeding efficiency of HaCaTs on EDRT and IDRT (*** p<0.001).
HaCaTs were seeded onto the top surface of EDRT and IDRT to examine cell-scaffold interactions including cell attachment, proliferation and migration. SEM analysis revealed HaCaTs attached to the surface of both scaffolds within 24 hrs and began to form bridges between cells covering the interfibre spaces (Figure 4.12, orange arrows). These bridges suggested that EDRT and IDRT have favourable surfaces which can support HaCaT attachment and migration to form a confluent layer (342). Formation of a subconfluent cell layer likely occurred through cell migration rather than cell proliferation as the culture period was too short for exponential cell proliferation to have begun (367). By day 14 post-seeding, HaCaTs had formed confluent layers on the scaffold surfaces with no cellular bridges or scaffold pores evident. EDRT also had the characteristic rough surface of keratinocytes. This rough surface was likely due to the ongoing development of an epithelial layer of HaCaTs forming a differentiated...
epithelium. This behaviour has been previously observed on collagen gels *in vitro* (282).

![Figure 4.12: SEM images of cellular bridges (orange arrows) on the top surface of HaCaT-seeded EDRT (left column) and IDRT (right column) at day 1 (A and B) and day 14 (C and D) post-seeding. Magnification is 1000× and scale bars are 10 μm.](image)

In H&E-stained cross-sections, cells appeared as purple spots (Figure 4.13, orange arrows). A partial layer of HaCaTs was observed on the surface of EDRT on day 1 post-seeding (Figure 4.13A); however, on day 14 no cell layer was found (Figure 4.13B and C) and there was also no cell layer observed on IDRT at all-time points (Figure 4.13B, D and F).
Figure 4.13: H&E staining of cells stained purple (orange arrows) and tissue deposition remnants (green arrows) on HaCaT-seeded EDRT (left column) and IDRT (right column) at day 1 (A and B), day 14 (C and D) and day 28 (E and F) post-seeding. Magnification is 20× and scale bars are 200 μm.

This lack of cell layer is suspected to result from separation of the cells from the scaffolds during processing, especially since layers of HaCaT cells were observed in
SEM images (Figure 4.12). This cell layer separation was previously observed in CEP scaffolds (Figure 3.27A and B) and in these H&E images there appear to be remnants of tissue deposition (Figure 4.13, green arrows). However, separation cannot be confirmed for EDRT and IDRT as there were images showing this occurrence.

In both H&E and DAPI imaging HaCaTs were observed inside the scaffolds and DAPI staining revealed HaCaTs were more concentrated at the surface of EDRT in comparison to an even distribution of HaCaTs throughout IDRT (Figure 4.14). This could be attributed to migration of HaCaTs toward the surface of EDRT as elastin peptides, possibly generated from cellular elastin breakdown, can have chemotactic effects on keratinocytes (360).
With increased pore size, EDRT was expected to show higher dispersal of HaCaTs but surprisingly, EDRT appeared to have better localisation of HaCaTs at the scaffold surface. These results indicate the presence of elastin may have been responsible for
promoting HaCaT attachment, migration and/or cellular interactions thereby enabling the formation of an epithelial layer. The proliferation study showed no difference in cell numbers further indicating that the locations of the HaCaTs were critical to the formation of the epithelial layer as similar cell numbers alone on IDRT was insufficient. The accelerated development of an epithelial layer on EDRT is an encouraging result as this could translate into faster re-epithelialisation and wound healing \textit{in vivo}.

4.3.4 HDF behaviour on EDRT and IDRT

Seeding of HDFs on EDRT had $120 \pm 7.99\%$ efficiency in comparison to IDRT which had a cell-seeding efficiency recorded at $92.78 \pm 6.79\%$ (Figure 4.15); however, there was no significant difference observed in the data. HDF proliferation was monitored over 28 days with cell numbers consistently increasing on EDRT over 28 days from $229,645 \pm 27,351$ cells on day 7, $325,026 \pm 32,765$ cells on day 14 to $342,962 \pm 18,022$ cells on day 21 and $491,480 \pm 35,294$ cells on day 28 (Figure 4.16). In contrast, IDRT showed a reduction of cell numbers from days 7 to 21 with a drop in cell number from $293,000 \pm 46,500$ cells on day 7 to $254,666 \pm 41,353$ cells on day 14 and then to $233,639 \pm 394$ cells on day 21. However, an increase in cell number was observed from day 21 to $300,859 \pm 26,521$ cells on day 28 (Figure 4.16). EDRT was also observed to have significantly higher numbers of HDFs than IDRT on days 21 and 28.
Figure 4.15: HDF-seeding efficiency on EDRT and IDRT.

Figure 4.16: HDF proliferation over 28 days on EDRT and IDRT (* p < 0.05, ** p < 0.01) (366).
The improved proliferation of HDFs on EDRT could be attributed to the inclusion of tropoelastin during fabrication, which can promote cell interactions as shown in a previous study (368). Other studies have shown that a larger surface area to volume ratio, as seen in EDRT, can also be conducive to promoting cell proliferation (257). Significantly increased cell numbers on EDRT are likely to yield faster ECM production and remodelling in vivo thereby accelerating wound healing and take of skin grafts in burn survivors.

HDF interactions with EDRT and IDRT were also investigated by droplet-seeding HDFs onto the scaffolds and examining seeded-scaffolds. Using DAPI staining, HDFs were seen to infiltrate both scaffolds on day 7 (Figure 4.17A and B). Cells migrated through EDRT to approximately 250 μm on day 7 (Figure 4.17A). In contrast, most of the HDFs remained on the seeded surface of IDRT on day 7 (Figure 4.1B). On days 14 and 21 post-seeding, cells had distributed through the entire EDRT scaffold (Figure 4.17C and E) but on IDRT remained near the surface with a few cells migrating 200 μm into the scaffold (Figure 4.17D and F). This enhanced infiltration in EDRT could be attributed to the presence of elastin in the scaffold providing biosignals and altering structure. It has been observed that interconnecting pores and open interfibre spaces are key to controlling cell migration in addition to influencing tissue ingrowth, nutrient supply to cells, metabolic dispersal, local pH stability and cell signalling (369). Therefore, the significantly enlarged pore size and higher porosity observed in EDRT could have promoted cell infiltration and migration. Although, previous studies have shown the optimal pore size for HDF attachment and migration was smaller than 160 μm in PLA and PLGA scaffolds (274), these results show that enhanced cell infiltration was attainable on EDRT with pore diameters of 375.0 ± 48.4 μm.
Figure 4.17: DAPI images of HDFs (orange arrows) on HDF-seeded EDRT (left column) and IDRT (right column) at day 7 (A and B), day 14 (C and D) and day 21 (E and F) post-seeding. Magnification 10× and scale bars are 50 and 100 μm (366).
4.3.4.1 **HDF-induced contraction of EDRT and IDRT**

HDF infiltration and proliferation on scaffolds can cause scaffold contraction. Therefore, scaffold area of EDRT and IDRT was measured over 12 weeks. No contraction was observed over the short-term 28 day period (Figure 4.18) or long-term 12 week period (Figure 4.19) for both scaffolds. Surprisingly, EDRT and IDRT swelled in cell culture media and expanded up to 15 % of its original size for EDRT and 8 % of its original size for IDRT. This lack of HDF-induced contraction could be due to a lack of fibroblasts differentiating into contractile myofibroblasts *in vitro*. These processes usually follow inflammatory events (370) which are absent *in vitro*.

![Figure 4.18: Scaffold contraction of EDRT and IDRT during HDF culture over 28 days (366).](image)

*Figure 4.18: Scaffold contraction of EDRT and IDRT during HDF culture over 28 days (366).*
4.3.5 Co-culture of HaCaTs and HDFs on EDRT and IDRT

HaCaTs were seeded onto the same side of scaffolds as HDFs one week after seeding of HDFs. SEM analysis of scaffolds at day 1 showed formation of a cell layer with migrating cells observed on EDRT (Figure 4.20A, orange arrows); however, HDFs could not be distinguished from HaCaTs. On day 14, a flat cell layer was observed with a rough appearance (Figure 4.20C and D), which as described in the HaCaT culture, and is likely due to a developing epithelial layer (282). The small gaps in the cell layer on day 14 (Figure 4.20C and D, yellow arrows) confirms the presence of epithelial bridges as cells are subjected to high tension and when cell division occurs this can result in holes forming (342). By day 28 the surfaces of the cell layers were clearly rough, more so on EDRT than IDRT (Figure 4.20E and F), which is consistent with the more advanced epithelial layer development previously observed on EDRT in the HaCaT culture (Figure 4.12).
H&E analysis of cross-sections confirmed a confluent layer of cells on both scaffolds at day 1 (Figure 4.21A and B, orange arrows); however, on days 14 and 28 scaffolds appeared to have had some cells removed from the surface as deposited tissue was observed (Figure 4.21, green arrows) on sections of the scaffold surface with some cells present. DAPI staining confirmed the presence of cells at the surface of the scaffold and more clearly highlighted some cell infiltration on all scaffolds at all-time points (Figure 4.22). The cell infiltration is unlikely to result from active cell migration into the scaffold as the depth of infiltration and number of cells appears consistent throughout the 28 days indicating it was more likely the cells fell through the pores during seeding.

Epithelial development appeared more advanced in the co-culture compared to the HaCaT culture with a rougher surface observed. This improvement is in accordance with previous studies which have shown both cell types are necessary for the development of normal epidermal morphogenesis (371, 372). Further analysis of cell distribution via cell specific stains could not be conducted due to separation of the cell layer from the scaffolds during sample preparation or from loss of cell attachment to the scaffold surfaces.
Figure 4.20: SEM images of migrating cells (orange arrows) and gaps in the cell layer (yellow arrows) on the top surface of HaCaT- and HDF-seeded EDRT (left column) and IDRT (right column) at day 1 (A and B), day 14 (C and D) and day 28 (E and F) post-seeding. Magnification is 1000× and scale bars are 10 μm.
Figure 4.21: H&E images of confluent cell layers (orange arrows) and deposited tissue (green arrow) on HaCaT- and HDF-seeded EDRT (left column) and IDRT (right column) at day 1 (A and B), day 14 (C and D) and day 28 (E and F) post-seeding. Magnification is 20× and scale bars are 200 μm.
Figure 4.22: DAPI images of cells (orange arrows) on HaCaT- and HDF-seeded EDRT (left column) and IDRT (right column) at day 1 (A and B), day 14 (C and D) and day 28 (E and F) post-seeding. Magnification is 10× and scale bars are 400 μm.
4.4 Summary

The inclusion of tropoelastin in the commercial dermal substitute, IDRT, to form EDRT had no effects on integrity of the scaffolds while significantly enlarging average pore diameter, increasing porosity and increasing deformability. EDRT also displayed more advanced epithelial development, accelerated HDF infiltration and improved HDF proliferation \textit{in vitro}; however, similar to the electrospun CEP scaffolds there was no significant degradation of IDRT or EDRT observed during the study period. Overall, EDRT has great potential as a novel dermal substitute to improve elasticity and accelerate healing of severe burn injuries.
Chapter 5

Animal studies of novel, skin substitutes
Chapter 5  Animal studies of novel, skin substitutes

5.1 Introduction

Safety and efficacy of a skin substitute is of the utmost importance. The effects and outcomes of a substitute on the wound, and the body, must be predictable. Without such predictability, a substitute cannot be responsibly used in treatment. As such, a crucial step in the development of skin substitutes is the investigation of their performance in a controllable environment. Unfortunately, there are no in vitro systems which accurately replicate the human system. Therefore, mammalian animal models representative of the human system are utilised to investigate potential, skin substitutes and examine host response to substitute properties such as the materials used for fabrication. Common models employed in skin tissue engineering are mouse, rat and pig models.

Mouse models are frequently utilised to study skin substitutes as they are economical, handling of mice requires little training and mice are relatively easy to house and maintain. The structure and the function of mouse skin are similar to humans with an outermost epidermis and inner dermis which is attached to adipose tissue (Figure 5.1). However, there are several points of difference such as: the epidermis of mice is much thinner than humans with fewer cell layers; the dermis is also thinner and lacks rete ridges; mice have more densely packed hair follicles; and immune systems in mice contain different cells such as Mast and γδ T cells (373) (Figure 5.1). In addition to this, wound healing in mice occurs primarily by contraction as the skin is loosely connected to the subcutaneous connective tissue whereas humans heal by
re-epithelialisation due to firmly-attached skin (Table 5.1). These differences can result in responses which vary from humans but for use in characterisation of substitutes, and first in vivo assessment of biosafety and efficacy, mouse models are ideal.

Figure 5.1: Schematic of mouse skin (A) and human skin (B) illustrating differences in structure and immune cell types (373).
Table 5.1: Comparison between human, mouse and pig skin anatomy and physiology. Adapted from (374).

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Human</th>
<th>Mouse</th>
<th>Pig</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin attachment</td>
<td>Firmly attached</td>
<td>Loosely attached</td>
<td>Firmly attached</td>
</tr>
<tr>
<td>Hair coat</td>
<td>Sparse</td>
<td>Dense (except some breeds)</td>
<td>Sparse</td>
</tr>
<tr>
<td>Epidermis</td>
<td>Thick</td>
<td>Thin</td>
<td>Thick</td>
</tr>
<tr>
<td>Dermis</td>
<td>Thick</td>
<td>Thin</td>
<td>Thick</td>
</tr>
<tr>
<td>Panniculus carnosus</td>
<td>Absent</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>Healing mechanism</td>
<td>Re-epithelialisation</td>
<td>Contraction</td>
<td>Re-epithelialisation</td>
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Pig model studies commonly follow on from mouse model studies as a tool to further, and more accurately, characterise potential skin substitutes. The structure of pig skin has similar thickness of the epidermal and dermal layers to human skin; however, the adipose tissue layer is generally thicker than in humans (374). Further similarities arise in the wound healing mechanisms with wound healing in pigs proceeding via re-epithelisation as pig skin is also firmly attached to the subcutaneous layer as seen in humans (Table 5.1). Despite the similarities between human skin and pig skin, pig models are used at a later stage of characterisation due to the expense incurred.
5.1.1 Established mouse models

Different mouse models have been established to examine potential, skin substitutes. One study used a subcutaneous implant model in Balb/c mice to investigate host response to an electrospun, CE scaffold (273). Scaffolds were implanted through 1 cm incisions in the dorsal area of male and female mice and skin biopsies were collected 6 weeks post-implantation for histological analysis. This model allowed visualisation of responses such as mild inflammation, cell infiltration, ECM protein deposition and angiogenesis.

A substrain of Balb/c mice, called Balb/c nude, have been used in another study to examine a living, skin substitute (375). This strain of mouse is immunodeficient and was specifically chosen as human cells were used in the construction of the living substitute using acellular, dermal matrix of porcine origin. Hair follicle stem cells or immortalised bulge stem cells formed the epidermal layer and dermal papilla cells or dermal fibroblasts formed the dermal layer. Full-thickness, skin wounds were excised on the dorsal area of each mouse and substitutes grafted using an auto-adhesive, polyurethane, transparent bandage. This model revealed the development of embryonic, hair bud-like structures from human, dermal papilla cell-containing constructs in vivo without immune rejection. Other studies have also used skin excision procedures in athymic mice: one to evaluate mechanical properties, such as tensile strength and elasticity, by uniaxial tensile testing of substitutes after grafting (376); and another to examine wound contraction of scaffolds produced using different techniques by photographing and tracing wounds which were quantified using computer planimetry (278).
Another model of full-thickness, skin excision grafting has been examined in Balb/c and C57BL/6 mice in which wounds are splinted to prevent wound healing by contraction (377). This results in a healing process more like humans in which new tissue is generated through granulation and re-epithelialisation as skin is tethered to subcutaneous tissue (378-381). Wound closure can be photographed, as in non-splinted wounds, with variations in contraction arising from skin contraction, animal posture and motion and wound dressing minimised (377) and after transplantation of living, skin substitutes cells can also be tracked via markers such as fluorescent protein gene (378, 382) or fluorescent dye (383).

The use of mouse models is common and the choice of model depends on the characterisation required: subcutaneous models are ideal for examining host responses such as inflammation, immune response and cell-scaffold interactions to new substitutes; non-splinted grafting is sufficient for analysis of cytokines and gene or protein expression; and splinted grafting is useful for examination of wound closure in an environment more like human wound healing.

5.1.2 Aims

The previous studies investigated the morphological, mechanical and cell-interactive properties of two electrospun, CEP scaffolds in Chapter 3 and lyophilised EDRT in Chapter 4. The aim of this study was the characterisation of these novel, scaffolds using two established models: firstly, a subcutaneous implant, mouse model was chosen for examining the biosafety and efficacy of the electrospun and lyophilised scaffolds in wound healing by examining ECM deposition, scaffold remodelling and collagen and elastin gene expression; and secondly a full-thickness skin excision model was used to assess contraction and angiogenesis in EDRT.
5.2 Methods

Please refer to section 2.2.3 for all protocols used in this study.

5.3 Results and Discussion

5.3.1 Host response to CEP 1 and CEP 2 in an established, subcutaneous implant model in mice

Electrospun scaffolds were examined in vivo using an implantation model with skin biopsies collected weeks 1, 2, 4, 12 and 24 post-surgery. H&E staining of cross-sections visualised cell infiltration of scaffolds by staining nucleic acids purple and scaffolds pink or light purple. Cells completely infiltrated both CEP 1 and CEP 2 at week 1 post-surgery (Figure 5.2A and B, purple spots) and vascularisation was observed in CEP 1 at 2 weeks with blood cells staining bright pink (Figure 5.2C, black arrows). By week 4, CEP 1 was fragmented (Figure 5.2E, green arrows), comparatively CEP 2 retained scaffold structure with no fragmentation (Figure 5.2F). No further degradation of CEP 1 fragments was seen at weeks 12 and 24 post-surgery (Figure 5.2G and J).
Figure 5.2: H&E images of pink blood vessels (black arrows), scaffold fragments (green arrows), inflammation (circled in green) and scaffold remodelling (circled in black) on implanted CEP 1 (left column) and CEP 2 (right column) at week 1 (A and B), week 2 (C and D), week 4 (E and F), week 12 (G and H) and week 24 (J and K) post-surgery. Magnification is 20× and scale bars are 100 μm.
The rapid vascularisation of CEP 1 seen at 2 weeks post-surgery is an encouraging result for the treatment of full-thickness burn wounds which characteristically lack sufficient blood vessels; however, CEP 1 also underwent rapid scaffold fragmentation with no remodelling observed. Comparatively, no vascularisation of CEP 2 was observed over the 24 week period and scaffold remodelling was evident at 12 weeks (Figure 5.2H, circled in black) with tissue deposition observed at the edges and small pockets of tissue stained pink throughout the scaffold. Scaffold remodelling became more evident at 24 weeks with remodelling observed in the middle of the scaffold (Figure 5.2K, circled in black). Limited scaffold degradation was likely due to the high presence of elastin preventing replacement by tissue due to elastin’s long half-life of approximately 70 years (183). No inflammation was observed in both scaffolds at week 1 (Figure 5.2A and B), but there appeared to be minimal inflammation at week 2 in CEP 2, indicated by dense purple staining of cells (Figure 5.2D, circled in black).

Masson’s Trichrome staining of the skin biopsies stained all collagens turquoise, nucleic acids purple and muscle and blood cells bright pink. Presence of scaffold collagen could be seen at weeks 1 and 2 post-surgery with a turquoise hue to the scaffolds (Figure 5.3 A-D). At week 2 post-surgery collagen degradation appeared to have occurred with the turquoise stain slightly fainter (Figure 5.3C and D). The inferred degradation of scaffold rat tail collagen could account for the minimal inflammation previously observed in H&E stained sections (Figure 5.2D). By weeks 4 and 12 the turquoise hue was less pronounced indicating a further decrease in scaffold collagen likely due to continued degradation (Figure 5.3 E-H). By week 24 the purple staining of the scaffold fibres indicated there was no collagen present in scaffolds which could be due to the scaffold structure of CEP 1 and CEP 2 providing sufficient structural support for cells to the extent that tissue deposition by infiltrated cells was unnecessary.
(Figure 5.3J and K). However, the persistence of the scaffold indicates the scaffold was not degraded due to the high elastin content. Collagen deposition was observed from week 4 onwards in tissue encapsulating both scaffolds (Figure 5.3E and F) and, more importantly, in newly deposited tissue from scaffold remodelling of CEP 2 at weeks 12 and 24 stained a faint turquoise (Figure 5.3H and K, circled in black).
Figure 5.3: Masson Trichrome images of scaffold remodelling (circled in black) on implanted CEP 1 (left column) and CEP 2 (right column) at week 1 (A and B), week 2 (C and D), week 4 (E and F), week 12 (G and H) and week 24 (J and K) post-surgery. Magnification is 20× and scale bars are 100 μm.
IHC staining for elastin was performed to visualise elastin as a brown stain and thereby examine the presence of elastin. The bovine elastin used to produce the electrospun scaffolds was observed at almost all-time points in CEP 1 except at week 4 (Figure 5.4 left column). The lack of elastin at week 4 is likely due to rapid fragmentation and clearance of the CEP 1 fragments since elastin has a half-life of approximately 70 years (384). This also indicates CEP 1 had varying rates of scaffold degradation in vivo since scaffold elastin was observed in skin biopsies collected at weeks 12 and 24. The rapid and variable degradation rate of CEP 1 is extremely undesirable in the treatment of burn injuries since persistence of the scaffold would be unpredictable and provide insufficient time for wound repair. Comparatively, elastin was observed at all-time points in CEP 2 indicating the structure of CEP 2 is more stable allowing for scaffold degradation and remodelling to transpire concurrently.

There were very small amounts of elastin observed in newly deposited tissue at week 4 from encapsulation but this was unsurprising as a previous study found regeneration of fibres of elastin was not observed until up to several years after treatment (385).
Figure 5.4: IHC elastin images of implanted CEP 1 (left column) and CEP 2 (right column) at week 1 (A and B), week 2 (C and D), week 4 (E and F), week 12 (G and H) and week 24 (J and K) post-surgery. Magnification is 20× and scale bars are 100 μm.
Further analysis of the impact of CEP 1 and CEP 2 after implantation was conducted by examining gene expression of collagen and elastin. Of the different collagens found in skin, collagen type I was investigated as it is the major isotype of skin dermis (386) and the gene encoding the α1 chain, COL1A1, was chosen for qPCR analysis. Collagen type I expression had no significant differences between CEP 1 and CEP 2 at all-time points and expression was consistent over the 24 week period in both scaffolds (Figure 5.5). There was also no difference in elastin expression between the two scaffolds at all-time points but there was a significantly higher level of expression at week 4 compared to week 1 in both scaffolds (p<0.05) and higher non-significant elastin expression at 12 and 24 weeks (Figure 5.6). The increased elastin expression at week 4 could be attributed to the presence of elastin degradation products stimulating elastin expression as scaffold breakdown was observed from 2 weeks onwards in CEP 1 and 4 weeks onwards in CEP 2 (Figure 5.4). This is in accordance with a previous studies which observed increased elastin synthesis by pulmonary fibroblasts with damaged ECM (387) and also that the presence of enzymatic elastin products can have stimulatory effects on elastin production (187).
Figure 5.5: Gene expression analysis of COL1A1 by qPCR in skin biopsies of CEP 1 and CEP 2 at weeks 1, 2, 4, 12 and 24 post-implantation (n=2-4).

Figure 5.6: Gene expression analysis of elastin by qPCR in skin biopsies of CEP 1 and CEP 2 at weeks 1, 2, 4, 12 and 24 post-implantation (* p<0.05) (n=2-4).
5.3.2 Host response to EDRT and IDRT in an established, subcutaneous implant model in mice

Cell interactions on lyophilised scaffolds were examined using the subcutaneous model previously described for the electrospun scaffolds. Skin biopsies were collected weeks 1, 2, 4, 12 and 24 post-surgery with H&E staining performed to visualise cell distribution throughout the scaffolds. Purple-stained cells could be seen distributed throughout both EDRT and IDRT at week 1 with new tissue deposition evident by pink webs of fine fibres in between thicker scaffold fibres (Figure 5.7A and B). By week 2 more cells were located in both scaffolds with increased amounts of tissue deposition (Figure 5.7C and D). From week 4 onwards, tissue deposition progressively increased with similar amounts of tissue deposition in both scaffolds at 24 weeks with dense webs of fine, pink fibres observed (Figure 5.7E-K).
Figure 5.7: H&E images of implanted EDRT (left column) and IDRT (right column) at week 1 (A and B), week 2 (C and D), week 4 (E and F), week 12 (G and H) and week 24 (J and K) post-surgery. Magnification is 20× and scale bars are 100 μm.
Masson’s Trichrome staining confirmed the presence of scaffold collagen in both EDRT and IDRT with thick scaffold fibres staining turquoise and no collagen degradation observed at all times points (Figure 5.8). Newly deposited tissue in weeks 1 and 2 post-surgery did not appear to contain collagen with fine fibres staining purple (Figure 5.8A-D), but at week 4 fine fibres had a turquoise tinge indicating the deposition of collagen by cells as part of the new ECM (Figure 5.8 E and F). It is likely collagen deposition had begun to occur earlier, since cells begin to remodel wounds after a few days (388), but the amount of collagen was too small to be viewed by microscopy. New tissue was clearly staining turquoise at weeks 12 and 24 showing increased collagen content at later time points (Figure 5.8G-K). Some vascularisation of EDRT was observed at week 2 and in IDRT at week 4 with blood cells staining bright pink. Angiogenesis in EDRT and IDRT was further examined in vivo using a one-step grafting model as described later in section 5.3.3.1. Gene expression analysis of COL1A1 confirmed the consistent expression of collagen over the 24 week period with no significant differences observed between EDRT and IDRT or between weeks (Figure 5.9).
Figure 5.8: Masson Trichrome images of implanted EDRT (left column) and IDRT (right column) at week 1 (A and B), week 2 (C and D), week 4 (E and F), week 12 (G and H) and week 24 (J and K) post-surgery. Magnification is 20× and scale bars are 100 μm.
In addition to collagen, the ECM protein elastin was investigated using IHC with elastin appearing brown. As expected fibres of EDRT stained brown at all-time points confirming the presence of elastin in the EDRT scaffold whereas IDRT stained a faint purple from haematoxylin counterstaining due to absence of elastin. Elastin deposition was not observed in either scaffold at week 1 nor in EDRT at week 2 (Figure 5.10A-C). IDRT displayed a few pockets of elastin deposition week 2 post-surgery (Figure 5.10D) and by week 4 new tissue deposited in both scaffolds had a slight brown tinge, which was evident by colour comparison of new, fine fibres to thicker IDRT fibres (Figure 5.10E and F). By week 12 the faint brown hue of the fine fibres in EDRT and IDRT was more evident and became stronger at week 24 (Figure G-K). The increasing brown hue of the new tissue from weeks 4 to 24 is likely due to continual deposition and thereby accumulation of elastin.

![Figure 5.9: Gene expression analysis of COL1A1 by qPCR in skin biopsies of EDRT and IDRT at weeks 1, 2, 4, 12 and 24 post-implantation.](image)
Figure 5.10: IHC elastin images of implanted EDRT (left column) and IDRT (right column) at week 1 (A and B), week 2 (C and D), week 4 (E and F), week 12 (G and H) and week 24 (J and K) post-surgery. Magnification is 20× and scale bars are 100 μm.
The presence of elastin in IDRT at 2 weeks, and absence in EDRT, is surprising. Previous studies have shown the presence of elastin degradation products can promote the expression of elastin probably as a natural response to detecting damage to the ECM and facilitating repair (387). The apparent lack of newly synthesised elastin in EDRT at 2 weeks could simply be due to the thick, brown fibres of EDRT overwhelming faint, brown staining of new tissue since elastin gene expression analysis showed elastin expression was consistent over the 24 week period with no significant differences observed between EDRT and IDRT or between weeks (Figure 5.11). In contrast, despite the lack of significance there did appear to be a sustained increase in elastin expression from 4 weeks onwards in EDRT and 2 weeks onwards in IDRT, as seen in the IHC staining. These concurring results strongly indicate elastin expression was possibly affected by other factors, such as tumour necrosis factor-α, interleukin1β or insulin-like growth factor-1 (389), more than the presence of elastin degradation products.
5.3.3 Wound contraction and angiogenesis of EDRT- or IDRT-grafted, full-thickness, skin excision wounds in established grafting models in mice

5.3.3.1 Two-step grafting of mice

A two-step grafting procedure is a commonly used clinical practice for grafting of the bilaminar IDRT which requires a second procedure for the epidermal graft. This two-step grafting procedure was performed on mice to examine wound healing and contraction.

The procedure was mimicked by creating full-thickness, skin excisions on the dorsal area of mice. The wounds were grafted with EDRT or IDRT and covered with a silicone
layer (Figure 5.12) then dressed for 1 week. Despite daily monitoring, mice removed dressings and silicone layers resulting in severe drying grafts (Figure 5.13). Skin irritation during wound healing is encountered in clinic as well; however, it is possible to reason with or prevent patients from removing dressings and grafts. To overcome this issue in mice a one-step grafting of EDRT and single-layer IDRT was performed as described in the following section 5.3.3.2.

Figure 5.12: Image of EDRT and IDRT grafting on mice for two-step grafting procedure. Image of EDRT graft covered by a silicone layer (A) and image of IDRT (I) and EDRT (E) grafts on dorsal area covered by silicone (B).
5.3.3.2 One-step grafting of mice

One-step grafting of skin substitutes is currently performed in clinic with products such as MatriDerm. This procedure is similar to the two-step grafting procedure described above. The wound is still debrided and then grafted with the dermal substitute; however, the split-thickness autograft, which forms the epidermal layer, is grafted immediately after the dermal substitute during the same procedure. Mimicry of the one-step grafting procedure was performed in mice by creating full-thickness, skin excisions on the dorsal area which were grafted with EDRT or single-layer IDRT followed by immediate grafting of an autograft. The wounds were dressed for 1 week and contraction monitored over 4 weeks with angiogenesis examined at 2 and 4 weeks post-surgery.

Examination of skin biopsies 4 weeks post-surgery showed cells infiltrated both EDRT and single-layer IDRT (Figure 5.14B and D respectively, black arrows) and with newly
deposited ECM evident by pink staining of fine fibres (Figure 5.14B and D). These results agree with those observed in the subcutaneous implant model in which cell infiltration and ECM deposition were also observed. Additionally, survival of autografts was observed at 4 weeks with skin grafts showing normal skin histology rather than dried scar tissue (Figure 5.14A and B). Mild inflammation in EDRT and single-layer IDRT was also seen with small, purple spots indicating the presence of immune cells in the skin graft (Figure 5.14 A and B).

![H&E image of one-step EDRT graft (left column) and IDRT graft (right column) at week 4 post-surgery. Magnification is 4× (A and B) and 20× (C and D) (366).](image)

Examination of wound contraction in one-step grafted wounds on day 3 showed that contraction of untreated, open wounds was 13.4 %, autografted wounds was 10.3 %,
EDRT grafted wounds was 11.8 % and IDRT grafted wounds was 16.8 % (Figure 5.15). By day 7 open wounds had higher contraction of 59.9 % compared to 35.7 % in autografted wounds, 23.8 % in EDRT grafted wounds and 27.7 % IDRT grafted wounds respectively. The prevention of contraction by autografts, EDRT and IDRT at day 7 post-surgery compared to open wounds was maintained over the 28 day period with 100 % contraction of open wounds at day 28 post-surgery and 38.1 %, 37.6 % and 38.9 % of autografts, EDRT and IDRT grafted wounds respectively. There was no significance observed in the data.

The similar wound contraction observed between wounds treated with EDRT and IDRT was unexpected due to the increased deformability observed in EDRT during mechanical tests. However, a previous study has shown an elastin-coated, collagen matrix also had similar wound contraction to a collagen-matrix while both matrices reduced wound contraction compared to an untreated, open wound (385).
Figure 5.15: Wound contraction of full-thickness, skin excisions wounds kept as an open wound or grafted with either an autograft, EDRT+autograft or IDRT+autograft in one-step grafting procedures over a 28 day period (366).

Although the presence of elastin in EDRT did not reduce wound contraction, angiogenesis of EDRT grafted wounds was significantly improved compared to open and autografted wounds. Vascularisation was assessed using a fluorescent probe \textit{in vivo} and radiant efficiency of the wounds was quantified (Figure 5.16). At 2 weeks post-surgery EDRT had significantly higher radiant efficiency of \( (236.15 \pm 6.10) \times 10^7 \), and therefore more vascularisation, compared to all other wounds with radiant efficiency of \( (21.43 \pm 4.58) \times 10^7 \) for open wounds, \( (26.23 \pm 7.98) \times 10^7 \) in autografts and \( (78.90 \pm 7.99) \times 10^7 \) for IDRT grafted wounds (\( p<0.01 \) compared to open and autografted wound and \( p<0.001 \) for IDRT grafted wounds; Figure 5.17). At 4 weeks, post-surgery vascularisation was similar between EDRT and IDRT grafted wounds, with \( (154.26 \pm 5.55) \times 10^7 \) and \( (149.15 \pm 6.45) \times 10^7 \) respectively, and wounds treated
with either EDRT or IDRT had significantly higher radiant efficiency compared to open, 
$(19.43 \pm 5.71) \times 10^7$, or autografted, $(16.13 \pm 5.27) \times 10^7$, wounds $(p<0.001)$ (Figure 5.17).

Figure 5.16: Representative images of four, full-thickness, skin excision wounds kept as an open wound (O) or grafted with an autograft (A), EDRT+autograft (E) or IDRT+autograft (I) in one-step grafting procedures for quantification of vascularisation at week 2 and week 4 post-surgery (366).
Figure 5.17: Quantification of vascularisation using fluorescent radiant efficiency of angiogenic probes in open wounds or wounds grafted with an autograft, EDRT+autograft or IDRT+autograft in one-step grafting procedures (** p<0.01, *** p<0.001) (366).

The significantly higher vascularisation of EDRT at 2 weeks confirmed the accelerated angiogenesis observed in Masson’s Trichrome staining (Figure 5.8). This is in accordance with a previous study in which elastin stimulated vascularisation (385) possibly due to the presence of elastin and its degradation products indicating a damaged environment (390). Staining of endothelial cells using marker CD146 confirmed vascularisation with small bundles of cells staining brown indicating the formation of new blood vessels in both EDRT and IDRT at 2 weeks (Figure 5.18, red arrows). The similar levels of vascularisation between EDRT and IDRT at 4 weeks could be due to binding or clearance of elastin degradation products in EDRT resulting in reduced signalling and therefore reduced vascularisation. The acceleration of angiogenesis in vivo by EDRT at 2 weeks could provide more nutrients and faster
exchange of waste products thereby facilitating wound regeneration. The vascular-inductive effect of EDRT could also be beneficial to the graft survival and wound healing of full-thickness burn wounds which can suffer from an insufficient vascular network.

Figure 5.18: IHC CD146 images of brown stained endothelial cells (red arrows) in one-step grafted EDRT (A) and IDRT (B) at week 2 post-surgery (366).

5.4 Summary

Histological analysis of implanted electrospun scaffolds showed CEP 2 had minimal inflammation with cell infiltration observed in the first week post-surgery. Scaffold remodelling and collagen deposition of CEP 2 were also seen from 4 weeks onward even though no scaffold remodelling was observed in vitro. However, in vitro experiments were performed for a shorter time of 4 weeks and the in vivo studies did not demonstrate scaffold remodelling at this time. In comparison, the performance of CEP 1 was far from ideal with rapid scaffold fragmentation and no remodelling observed. The reason for rapid fragmentation of CEP 1 was unclear and surprising since CEP 1 was previously shown to have higher energy loss compared to CEP 2 indicating a higher degree of cross-linking which should translate into a more stable
scaffold. Despite the differences in scaffold degradation rates between the two scaffolds, both upregulated elastin gene expression from 4 weeks onwards as well as having no negative impact on collagen gene expression. Of the two scaffolds, in vivo studies showed CEP 2 is promising as a skin substitute and further investigation regarding scaffold improvement and effects of ECM protein gene expression and synthesis is warranted.

Investigation of EDRT using a subcutaneous implant model showed new tissue deposition observed from 1 week post-surgery. Gene expression analysis of the ECM proteins collagen and elastin revealed consistent collagen expression was sustained over 24 weeks and elastin expression increased from 4 weeks onwards. Deposition of the collagen and elastin proteins could also be visualised in new tissue via staining at 4 weeks post-surgery with increasing protein accumulation observed at weeks 12 and 24. The presence of elastin in EDRT was also seen to significantly increase vascularisation at 2 weeks compared to IDRT with accelerated angiogenesis maintained at 4 weeks compared to open and autografted wounds. The modification of IDRT during fabrication to include 10 % (w/w) elastin yielded encouraging results, especially increasing early-stage angiogenesis which in turn could facilitate wound healing.
Chapter 6

General Discussion
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Over 11 million burn injuries require medical attention each year with partial- to full-thickness injuries often resulting in new tissue which lacks normal appearance and function. These undesirable results arise from skin substitutes which are unable to support skin regeneration. A serious, and unfortunately common, issue is inelastic skin due to missing or incorrectly deposited components of the ECM, particularly the protein elastin which plays a major role in the elastic network of skin. Elastin is often absent or fragmented in scar tissue and the inclusion of elastin during fabrication of two, novel scaffolds was examined in this thesis with the aim to improve elasticity and cell-scaffold interactions.

In this study the production of a novel, electrospun, CEP scaffold and the characterisation of this electrospun scaffold and EDRT, the elastin-modified version of the commercially-available IDRT, was performed. Characterisation of the scaffolds was accomplished by examining morphological, mechanical, cell-scaffold interactive properties \textit{in vitro} and host response in animal models.

6.1 Electrospun, CEP scaffolds

The materials and fabrication method utilised in the construction of a skin substitute have a significant impact on its performance and success. For this reason, electrospinning was chosen as the fabrication method as the resulting nano- to microscale fibres and final scaffold structure closely mimic native ECM (260) providing
a large surface area, which is favourable for cell attachment, and a highly porous scaffold to facilitate cell infiltration (260-262) while also reducing wound contraction compared to the more common fabrication method of lyophilisation (278). The three polymers used were chosen for their favourable results in tissue engineering: collagen is well-known for its biosignals and abundance in the ECM; elastin for its role imparting elasticity; and PCL for its strength and use in long-term implants with no negative side effects (199, 201).

Porous mats consisting of microfibres were successfully formed by electrospinning a combination of collagen, elastin and PCL and the four electrospinning parameters of polymer composition, flow rate, air gap and applied electric potential were investigated for their effects on electrospinning efficiency and morphological properties. The inclusion of 12 % (w/v) elastin during fabrication had the most notable effect with improved electrospinning efficiency of CEP scaffolds. CEP scaffolds remained intact and undamaged after cross-linking and tensile moduli of elastin-containing scaffolds was shown to be significantly lower than CP scaffolds indicating higher elasticity due to the presence of elastin. Elasticity is a favourable trait in skin substitutes as scaffolds will be better equipped to cope with shear stress.

Solution composition was also varied by increasing collagen content and simultaneously decreasing PCL content stepwise by 0.5 % (w/v) which had variable effects on electrospinning efficiency but did result in successively larger pores and wider fibres. Flow rate, air gap and applied electric potential also had variable effects on electrospinning efficiency. When one parameter was altered, with all others held constant, an improving or declining trend in electrospinning efficiency was observed resulting in the identification of a ‘sweet spot’ for fabrication. However, to acquire optimal conditions for scaffold fabrication alteration of one parameter would usually
require compensation by alteration of another parameter. Despite variances in electrospinning efficiency some trends were observed with morphological properties: increasing flow rate was seen to increase both pore diameter and fibre width and larger air gaps resulted in smaller pores and generally larger fibres.

Two cross-linked, electrospun, CEP scaffolds were chosen for their advantageous morphological and mechanical properties, specifically large pores, wide fibres and increased elasticity. CEP 1 was composed of the 1.5C12E1.5P solution and electrospun at 3 mL/h, 15 cm and 25 kV and CEP 2 was fabricated by electrospinning the 2C12E1P solution at 1 mL/h, 20 cm and 20 kV. Both scaffolds supported normal behaviour of HaCaTs *in vitro* with formation of a confluent, cell layer on day 1 and cell proliferation observed over 28 days. Normal HDF behaviour was also demonstrated with cell infiltration seen on days 14 and 28 with cell proliferation supported until day 21. Proliferation of both skin cell types was significantly higher on CEP 1 at various time points. HaCaTs also showed higher proliferation on CEP scaffolds due to their nature as a cell line compared to the short-lived primary HDFs which had slower proliferation. Therefore *in vitro* studies indicated CEP 1 as a more favourable skin substitute.

In contrast, *in vivo*, implant studies demonstrated more promising results with CEP 2. Both electrospun scaffolds supported cell infiltration from week 1 onwards with minimal inflammation observed at 2 weeks. CEP 1 underwent scaffold fragmentation from 2 weeks with no remodelling observed. Comparatively, remodelling of CEP 2 was observed at week 12 which became more pronounced at week 24. The new tissue deposited during remodelling in CEP 2 was shown to contain collagen but no newly synthesised elastin was observed. Despite the lack of observable elastin protein in the IHC elastin stain, gene expression analysis of elastin showed non-significant,
increasing expression from weeks 1-4 and sustained higher expression of elastin at weeks 12 and 24. This discrepancy between elastin gene expression and IHC staining of elastin protein is surprising and is likely due to the prominent brown staining of scaffold elastin overwhelming any newly synthesised elastin which would have stained a much fainter brown colour.

6.2 EDRT

EDRT is a modified version of the commercial, dermal, collagen-based substitute IDRT. Although IDRT is a popular dermal substitute wound contraction and scarring are still commonly encountered. EDRT was produced by lyophilisation using the same method as IDRT; however, in EDRT 10 % (w/w) of the collagen is replaced with recombinant, human tropoelastin for a final scaffold composition of 8 % shark chondroitin-6-sulphate, 10 % recombinant human tropoelastin and 82 % bovine, collagen type I. The inclusion of tropoelastin during fabrication, and thereby the presence of elastin after cross-linking, resulted in a scaffold with larger pores and higher porosity than its collagen counterpart, IDRT. The larger pores are thought to result from the increased elasticity of the scaffold, a desirable trait in a skin substitute. However, EDRT also demonstrated increased rigidity.

Cell studies revealed EDRT supported constant HaCaT and HDF proliferation over a 28 day period in vitro with no HDF-induced contraction observed. HaCaTs displayed normal behaviour forming and maintaining a confluent, cell layer with a rough surface characteristic of keratinocytes. Normal HDF behaviour was also seen with complete infiltration of EDRT compared to only partial infiltration of IDRT. The improved infiltration on EDRT can likely be attributed to its larger pores and the presence and function of elastin. However, despite cell infiltration no scaffold remodelling was
observed which could be due to the long half-life of elastin. As explained with the electrospun, CEP scaffold studies HaCaTs displayed higher proliferation as these cells originate from a cell line, whereas, HDFs are primary cells with a finite lifespan and variability.

Mouse model studies also displayed no scaffold remodelling over 24 weeks; however, new tissue deposition was visible from week 1 with no inflammation observed in both scaffolds. Collagen and elastin proteins were detected from 4 weeks onwards. Enhanced performance of EDRT was observed with accelerated angiogenesis 2 weeks post-grafting compared to IDRT.

6.3 Conclusions and future work

Electrospinning of the novel combination of collagen, elastin and PCL clearly demonstrated the ability of this fabrication method to produce structurally, biomimetic scaffolds capable of supporting remodelling in vivo. The synchronous temporal and spatial scaffold degradation and tissue deposition of CEP 2 in conjunction with the improved elasticity observed in vitro could decrease wound contraction. Additionally, scarring and contraction could also be further reduced because of the minimal and delayed inflammatory response caused by implantation of CEP 2. This short period of inflammation would indicate the wound does not have prolonged activity of TGF-β1 and TGF-β2 which leads to hypertrophic scarring and wound contraction (29). Investigations of TGF-β levels are warranted to elucidate this further. Following studies should examine the performance of CEP 2 as a living, skin substitute since translation of the formation of a keratinocyte layer in vitro to an in vivo setting could result in a substitute capable of supporting not only dermal, wound remodelling but re-epithelialisation as well. However, the lack of angiogenesis in implanted CEP 2
could result in metabolically inactive or even necrotic cells as the thickness of electrospun scaffolds could place keratinocytes further than 200 μm from a blood supply (271).

Comparatively, EDRT clearly demonstrated its capacity to act as a dermal substitute with its vascular inductive properties and deposition of new tissue inside pores despite a lack of scaffold degradation. Translation of this rapid angiogenesis would be particularly useful in full-thickness, burn injuries in which graft rejection or loss can occur due to insufficient vascularisation and therefore deficient nutrient supply and waste exchange. EDRT would be especially useful to replace single-layer IDRT which was specifically designed for use to help fill deep, burn injuries.

CEP 2 and EDRT are both promising, potential, skin substitutes and while further investigation would more clearly elucidate the properties of both scaffolds future work should consider producing a substitute which combines both scaffolds. A bilayered scaffold could result in a skin substitute capable of treating full-thickness burn injuries with a single substitute that would improve elasticity, accelerate angiogenesis, support dermal remodelling and serve as a template for re-epithelialisation.
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