# An alginate hydrogel matrix for the localised delivery of a fibroblast/ keratinocyte co-culture to expedite wound healing.

By

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# Abstract

There is a clinical need for effective alternative skin replacements to autografts, allografts and xenografts. In this thesis a bi-layer skin graft was developed by encapsulation of fibroblasts in calcium-alginate hydrogel and culture of keratinocytes on the surface. Initially, the use of 5% and 2% w/v alginate hydrogels were investigated. Both scaffolds maintained fibroblast viability for at least 150 days encapsulation and caused reversible mitotic and catabolic inhibition, as assessed by fluorescent staining, immunochemistry and the thiazolyl blue assay. Sustained expression of angiogenic factors such as vascular endothelial growth factor, interleukin 6 and nerve growth factor were seen by fibroblasts encapsulated in both scaffolds, by reverse transcription polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay. Histological staining demonstrated that following degradation of the scaffolds, fibroblasts secreted ECM to facilitate dermal repair. Comparison of degradation of the scaffolds over time by measuring release of calcium, and changes in rheological properties, morphology and mass, indicated that 5% w/v alginate hydrogel degraded more slowly and was preferable to 2% w/v alginate hydrogel. Fibroblasts encapsulated in 5% w/v alginate hydrogel were shown to express keratinocyte growth factor by RT-PCR, to support keratinocyte proliferation and differentiation, and keratinocytes cultured on the 5% w/v alginate hydrogel surface were seen to form multi-layered epidermal structures by histology, immunostaining and RT-PCR.

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# List of abbreviations

A/L – air liquid interface

ALP – alkaline phosphatase

Bcl - B-cell lymphoma

BM – basement membrane

BMSC – bone marrow stromal cell

BSC – basal cell carcinoma

Calcein-AM – calcein acetoxymethylester

CEA – cultured epithelial autograft

DAB – 3,3'-diaminobenzidine

DED – de-epidermalised dermis

DMEM – Dulbecco's modified Eagle's medium

DNA – deoxyribonucleic acid

EB – epidermal bullosa

EC – endothelail cell

ECCM – endothelial cell culture media

ECM – extracellular matrix

EDC - 1-ethyl-3-3-dimethylaminopropylcarbodiimide hydrochloride

EDTA – ethylenediaminetetraacetic acid

EGF – epidermal growth factor

ELISA – enzyme-linked immunosorbent assay

ESC – embryonic stem cell

FBS – foetal bovine serum

FCM – fibroblast culture media

FDA – US drug and food administration

FGF – fibroblast growth factor

GAG – glycosaminoglycan

GAPDH – glyceraldehyde 3-phosphate dehydrogenase

GF – growth factor

GM – CSF – granular macrophage stimulating colony factor

GVDH – graft versus host disease

HA – hylauronic acid

H&E – haematoxylin and eosin

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HVG – haematoxylin Van Gieson

HIF – 1 - including hypoxia-inducible factor

ICC – immunocytochemistry

ICP-MS – inductively coupled plasma mass spectroscopy

IGF – insulin-like growth factor

IHC – immunohistochemistry

IL – interleukin

IMS 99 - industrial methylated spirit 99%

INV – involucrin

JC-1 – 5,5',6,6'-tetrachloro-1,1',3,3'tetra-ethylbenzimidazolylcarbocyanine iodide

K – keratin

KCM – keratinocyte culture media

KGF- keratinocyte growth factor

mRNA – messenger ribonucleic acid

MSC – mesenchymal stem cell

MMP – matrix metalloprotein

MSH – melanin stimulating hormone

MTT – 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide

NGF – nerve growth factor

PanK – pan cytokeratin

PAP – peroxidase-anti-peroxidase

PBT - poly(butylenes terephthalate)

PCNA – proliferating cell nuclear antigen

PDGF – platelet derived growth factor

PET – polyethylene terephthalate

PEGT - poly(ethyleneglycol- terephthalate)

PBS – phosphate buffered saline

PI – propidium iodide

PLGA - poly(D,L)-lactide-co-glycolide

P/S – penicillin-streptomycin

RGD – arginine-glycine-aspartic acid

RT-PCR – reverse transcription polymerase chain reaction

SC – stratum corneum

SD – standard deviation

SDF – stromal cell-derived factor

SIS – small intestinal submucosa

SJS – Stevens Johnsons Syndrome

TAE – tris acetate EDTA

TBSA – total body surface area

TE – tissue engineered

TEN – toxic epidermal necrosis

TGF – transforming growth factor

UV – ultraviolet

VEGF – vascular endothelial growth factor

# 1. Introduction

There has been a consistent increase in the mean life expectancy of the population of the developed world over the past century. Healthy life expectancy, however, has not increased concurrently. As a result we are living a larger proportion of our lives in poor health (Christensen *et al.* 2009) and there is a growing demand for the replacement of diseased and damaged tissues (Langer & Vacanti 1993) including skin (Falanga 1993). Skin can be lost due to infection, trauma or disease (MacNeil 2007). Effective wound healing is essential to enable maintenance of homeostasis and to prevent penetration of infectious agents (Elias 2007; Menon & Kligman 2009). Skin can undergo spontaneous repair following damage, provided the affected area is less than 4cm in diameter (Herndon *et al.* 1989) and there is no underlying genetic abnormality or disease affecting wound healing (MacNeil 2007). In cases where wounds do not spontaneously heal, surgical replacement of the skin must be performed.

While autografts remain the 'gold standard' in skin replacement (Ben-Bassat *et al.* 2001), alternative treatments are necessary where there is inadequate donor site availability (Gajiwala & Gajiwala 2004) or underlying conditions affecting healing of secondary wounds produced by harvesting of autografts. Furthermore, tissue harvest and transplantation are associated with undesirable consequences such as the risk and expense of surgery, additional infection at the secondary wound site and rejection of the transplanted tissue (Atiyeh *et al.* 2005; Clark *et al.* 2007; Sahota *et al.* 2004). Alternatively, wound coverage can be provided by the use of allografts or xenografts, but these grafts are associated with disease transmission and immune rejection, making them only suitable as a temporary wound covering (Leon-Villapalos *et al.* 2010; Sahota *et al.* 2004). For this reason, there is a need for synthetic alternatives.

Skin is a complex tissue consisting of two main anatomically distinct regions known as the epidermis and the dermis. Following the establishments for the culkture of keratiocytes in vitro (Rheinwald & Green 1975a), the first tissue engineered (TE) skin consisted of a cultured epithelial autograft (CEA), with no dermal component which was developed in 1975 (Rheinwald & Green 1975b), and since then a number of CEAs have become commercially available. CEAs are designed to provide epidermal replacement to facilitate restoration of the barrier function of skin to prevent penetration of foreign materials and prevent heat and water

loss from the body (Gibbs & Ponec 2000; Liu *et al* 2007). CEAs have, however, been associated with various problems due to lack of a dermal component, including lack of take/ blistering, fragility, scarring and extensive wound contraction (Meana *et al.* 1998b). This has lead to the development of dermal grafts, which can be applied in conjunction with a CEA, and bi-layer composite grafts with both a dermal and epidermal components. Restoration of the dermal layer is essential (Caissie *et al.* 2006; Metcalfe & Ferguson 2007) since the dermis provides skin with mechanical integrity (Metcalfe & Ferguson 2007) and facilitates adherence of the epidermis to the body surface (Rennekampff *et al.* 1997).

At present, however, there are no commercially available composite grafts consisting of dermal and epidermal components together in one grafting stage that can provide permanent autologous skin replacement for full-thickness wounds (Ikada 2006). Furthermore, almost all the commercially available grafts with a dermal component include animal or human derived products such as collagen, glycosaminoglycans (GAGs) or deepidermalised dermis (DED), which can result in immunological responses and disease transmission. Alternative scaffold materials, from non-animal sources should be sought (MacNeil 2007).

In order that successful healing of full thickness wounds is achieved, it has been shown that the presence of both keratinocytes and fibroblasts is beneficial (Maas-Szabowski *et al.* 2000;Witte & Kao 2005). The aim of this project was to develop a composite bi-layer graft using a fibroblast/ keratinocyte co-culture with an alginate hydrogel scaffold. Alginate is a polysaccharide derived from algae which has shown great promise within the field of regenerative medicine, including the immuno-isolation of mammalian cells for the sustained release of therapeutic molecules (Bazou *et al.* 2008; Calafiore *et al.* 2006; Gao *et al.* 2005; Jen *et a.l* 1996; Soon-Shiong 1999), as a wound dressing (Balakrishnan *et al.* 2006; Boateng *et al.* 2008; Chiu *et al.* 2008; Lansdown 2002b), and also within tissue engineering (Augst *et al* 2006). Alginate is US Food and Drug Administration (FDA) approved for use in food and for many types of medical applications including use as a wound dressing and haemostatic agent.

In this thesis, the use of alginate hydrogel as a scaffold for the localized delivery of a fibroblast/ keratinocyte co-culture to engineer a skin bi-layer *in vivo* has been investigated. The construct should be delivered to the wound site and become integrated into the host tissue (Langer & Vacanti 1993). The potential of the tissue engineered bi-layer to become

successful integrated, including the regeneration of the dermal tissue by fibroblast proliferation, extracellular matrix deposition and vascularisation, and to facilitate epidermal regeneration was investigated.

In order that epidermal and dermal replacement is achieved, the scaffold should maintain the viability of encapsulated fibroblasts for extended periods of time. In order that the fibroblasts in the dermal layer remain viable, the metabolic demand of the encapsulated fibroblasts in the avascular graft should be low, and the fibroblasts should secrete angiogenic factors such as vascular endothelial growth factor (VEGF), nerve growth factor (NGF) and interleukin-6 (IL-6) to promote vascular in-growth. To achieve epidermal replacement the fibroblasts in the dermal layer should be mitotically inhibited so that they do not over grow the keratinocytes in the co-culture. The fibroblasts should also secrete factors such as keratinocyte growth factor (KGF) to support the proliferation and differentiation of keratinocytes in order that they can produce a stratified epidermal layer. The scaffold should also degrade in a controlled manner in order that cells remain immobilized at the site of implantation, where they can secrete ECM to replace the degrading scaffold material to achieve dermal regeneration.

# 2. Skin structure, functions and wounds

Skin is a complex structure consisting of multiple layer and cell types, which performs a number of functions. Skin wounds can occur as a result of disease, trauma, infection or injury. In order that the structure and function of skin is restored, it is essential that successful wound healing occurs. In this chapter, the structure and functions of skin are summarised, along with causes of skin wounds and the wound healing cascade.

## 2.1 Skin structure

The skin is a physiologically and anatomically specialised boundary lamina essential to life. The skin typically occupies almost two square metres of surface area, and accounts for 8% of the body's mass, making it the largest organ in the body. The thickness of skin varies widely depending on the part of the body in which it is found. For example, on the eyelids it is less than 0.5mm thick, whereas on the middle of the upper back it is more than 5mm thick, but, characteristically it is 1-2mm thick (Gray 1987; Carola *et al* 1990).

Skin is structurally complex and highly specialised, consisting of two intimately associated main layers called the epidermis, which is the outermost layer of keratinised squamous epithelial tissue, and the dermis, or corium, a thicker layer of connective tissue beneath the epidermis. Certain appendages to skin such as hair follicles and sweat glands span both the epidermis and the dermis, and penetrate into the subcutaneous adipose tissue below the dermis (Alberts *et al* 2002; Carola *et al*. 1990). The general architecture of skin is illustrated in Figure 2.1.

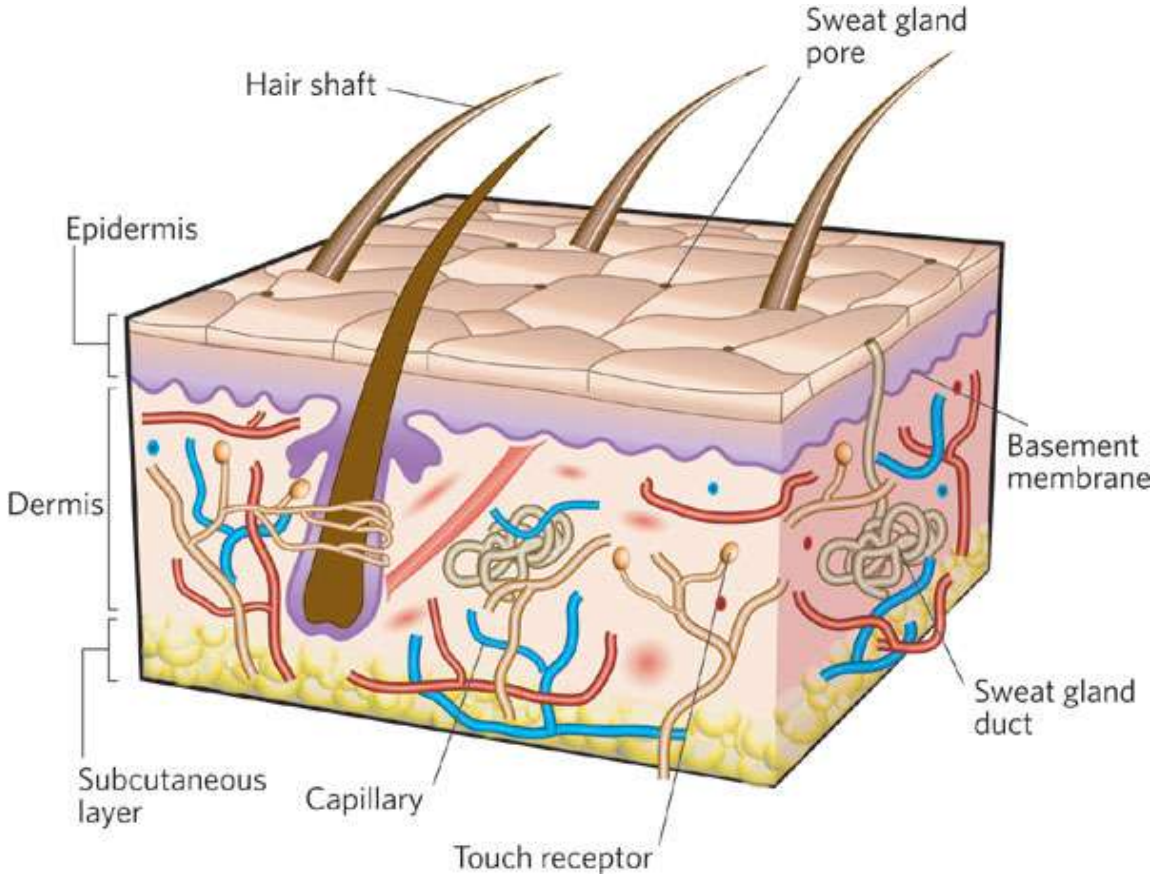



**Figure 2.1: Illustration showing the main components of skin (MacNeil 2007). The skin comprises of two main layers called the epidermis and the dermis, which are separated by a basement membrane. Below the dermis there is a subcutaneous fatty layer.**

### 2.1.1. Variation in Skin Structure

The appearance and function of specific areas of the integument can vary due to variations in the activity, frequency and types of glands, or size, distribution and shape of hairs and their follicles. In addition, the skin may vary due to local variations in thickness, mechanical strength, degree of keratinisation, pigmentation or vascularity. The appearance and structure of skin is not only due to genetics, but may also be affected by normal hormonal changes, age, state of health and other factors (Gray 1987).

Skin can be classified either as thin, hairy (hirsute) skin, which constitutes the majority of the body's surface, or, thick, hairless (glabrous) skin, which covers the palms, soles and flexor surfaces of the digits (Gray 1987).

### 2.1.2. Epidermis

The outermost layer of skin, called the epidermis composes of keratinised stratified squamus epithelium. It contains no blood vessels, and as a result it is usually possible to rub off dead cells of the epidermis without bleeding (Carola *et al.* 1990). The epidermis comprises mainly of keratinocytes, but also contains other cell types such as Langerhans cells and melanocytes (Alberts *et al.* 2002). Human epidermis thickness varies in thickness from 50µm on the eyelids to 1.5mm on the palms and soles (So *et al.* 1998).

#### 2.1.2.1. Epidermal appendages

The epidermis and epidermal appendages together are known as the integument. Certain appendages to skin such as hairs, hair follicles, glands and nails span both the epidermis and the dermis (Alberts *et al.* 2002). These structures are formed by the in-growth or other modifications of the epidermis (Gray 1987).

#### 2.1.2.2. Epidermal strata

In epidermal tissue there is a continuous replacement of keratinocytes at the surface from the base layer of mitotic cells (Fuchs 2007). The rate of replacement is typically between 45 to 75 days, although this can vary due to body position, epidermal thickness, and degree of skin abrasion, time of day, hormonal changes, age and many other factors. In particular, certain pathological conditions may alter the rate of replacement as, for example, in psoriasis where the rate is as little as 8 days, and thus the keratinocytes do not fully differentiate before reaching the surface (Gray 1987).

As keratinocyte stem cells differentiate and move up through the epidermal strata they undergo progressive changes in shape and content, eventually transforming from polygonal living cells to dead, flattened squames full of keratin. Traditionally, the term epidermal keratinisation applies to the final stage of keratinocyte differentiation in which cells are converted to tough, cornified squames. Cells of the epidermis, however, have a varied and changeable pattern of keratin filament chemistry which changes as keratinocytes mature. Keratins of the basal stem cells are different to those of young keatinocytes and it is only in the upper layers of the stratum spinulosum that the final types of keratin filament begin to emerge. The classic process of epidermal keratinisation, therefore, only describes the final stages of maturation in which dead squames fill with keratin and other proteins such as involucrin are formed (Gray 1987).

The epidermis is usually divided into a number of strata which each represent a different stage in keratinocyte maturation. In glabrous skin, the epidermis is thick and comprises of five layers or strata. The strata from superficial to deep are the stratum corneum, stratum lucidum, stratum granulosum (granular layer), stratum spinosum (spinous layer) and stratum basale. In hairy skin, however, the stratum lucidum is absent (Carola *et al.* 1990), and only four strata are found as illustrated in Figure 2.2.

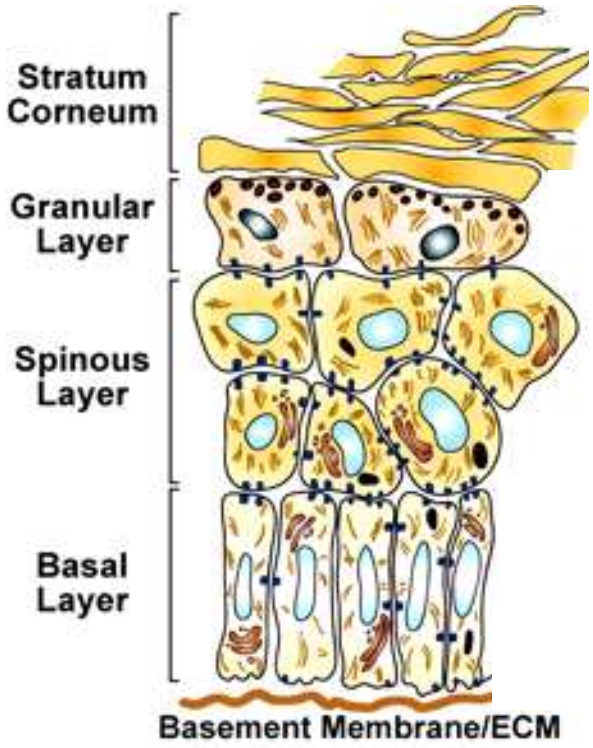



**Figure 2.2: General architecture of the epidermis of hairy skin (Fuchs 2008) showing the different strata representing keratinocytes at different stages of differentiation. The basal layer (stratum basal) lies upon the basement membrane, and above the basal layer there are the spinous and granular layers (stratum spinulosum and stratum granulosum, respectively) which together are known as the suprabasal layers. In glabrous skin a stratum lucidum is present above the granular layer. Finally, the stratum corneum forms the surface of the epidermis.**

#### 2.1.2.2.1. Stratum corneum

The stratum corneum is the horny, cornified layer of the epidermis, which as previously mentioned, is the outermost layer of epidermal tissue. It is a flat, relatively thick layer of

dead cells arranged in parallel rows. The cells in the corneum are constantly shed through normal abrasion, and are replaced by new cells formed by cell division and pushed up from the germinative layers below, which take on the function of the cells they replace (Carola *et al.* 1990). Depending on the location of the skin, this layer can vary from only a few cells thick, as in the scalp, to more than 50 cells thick. The cells are compact and contain high concentrations of keratin filaments each about 8-10nm thick, often lying parallel and 8nm apart, and embedded in another protein, filaggrin. Disulphide bond formation appears to stabilize this protein complex. A protein envelope, consisting of a number of cross-liked, chemically inert proteins including keratolinin and involucrin coats the internal surface of the cell membrane of the cells in this layer (Fuchs 2008; Gray 1987).

#### 2.1.2.2.2. Stratum lucidum

The stratum lucidum consists of flat, translucent layers of dead cells that contain the protein eleidin. This protein is thought to be a transitional substance between the soft keratin found in the corneum, and the precursor of soft keratin, keratohyaline, of the stratum granulosum. The lucidum only appears in glabrous skin, where it acts as a protective shield against the ultraviolet rays of the sun, thus preventing sunburn to these areas (Carola *et al*. 1990).

#### 2.1.2.2.3. Stratum granulosum

The stratum granulosum is usually two to four cells thick and lies just below the lucidum. The cells of this layer contain keratohyaline crystals. The layer initiates the process of keratinisation, associated with the dying process of cells (Carola *et al.* 1990).

#### 2.1.2.2.4. Stratum spinosum

The stratum spinosum is composed of several layers of mature keratinocytes known as polyhedral cells that have delicate ‘spines’ protruding from their surfaces, thus these cells are sometimes called “prickle cells”. The spine-like projections interlock with each other helping to give support to this binding layer. Cells of the spinosum layer grow and divide and new cells that are formed here are pushed to the surface to replace the cornified cells of the stratum cornium (Carola *et al.*1990).

#### 2.1.2.2.5. Stratum basale

The stratum basale rests on the basement membrane next to the dermis, and usually includes only a single layer of cells of columnar or cuboid appearance. Like the stratum spinosa, it too

is capable of undergoing cell division to produce new cells to replace those being sheared off in the exposed corneum layer (Carola *et al.* 1990). A population of stem cells found in this layer divide and differentiate, then move up and apart through the stratum spinulosum's lower region before ceasing mitotic activity (Fuchs 2009). Structurally the cells of the stratum basale are heterologous as they comprise both stem cells and keratinocytes in various stages of early maturation, as well as non-keratinocytes, such as melanocytes and Langerhans cells (Gray 1987).

### 2.1.3. Basement membrane

Under physiological conditions the epithelium is connected to the basement membrane, a specialised sheet-like ECM that forms an interface to mesenchymal tissues. Epithelial cells require adherence to a matrix for regular growth (Eckhart *et al*. 2003; Inoue *et al.* 2005).

The basement membrane of the epidermis consists of a basal lamina, about 80nm thick, to which all the cells of the stratum basale are attached, and on the dermal side, a reticular lamina which grades into the connective tissue of the dermis. The basal lamina of the skin consists of a lamina lucida lying close to the epidermal cell bases, and a deeper lamina densa. The lamina lucida is occupied by various macromolecules which gives this layer a finely granular or filamentous appearance. These macromolecules include heparan sulphate proteoglycan, laminin and a protein which is unique to skin called pemphigoid antigen. The lamina densa includes a network of type IV collagen molecules, a glycoprotein called epidermolysis bullosa acquisita antigen, fibronectin and various proteoglycans. Whilst the lamina lucida is strongly adherent to the overlying epidermal cell membrane, the meshwork of the lamina densa may limit the passage of macromolecules from the dermis to the epidermis. The basal lamina may also suppress the differentiation of keratinocytes in the stratum basale and regulate other cellular activities in the epidermis. A major function of the whole basement membrane is to mechanically stabilize the epidermis (Gray 1987).

### 2.1.4. Dermis

The dermis lies beneath the epidermis and constitutes the majority of skin. The cellular components of dermis include fibroblasts, endothelial cells, smooth muscle cells, and mast cells, but the bulk of dermis is made of extracellular matrix (ECM) (Supp & Boyce 2005b). The ECM consists of irregular, moderately dense, soft connective tissue consisting of an interwoven collagenous meshwork, mainly comprising of collagen type I, containing various

amounts of elastin fibres, proteoglycans, fibronectin, blood vessels, lymphatic vessels and nerves (Gray 1987).

The dermis comprises of two ill-defined layers, which are the thin papillary or subepithelial layer, which lies directly below the epidemis, and the deeper, thicker layer, called the reticular layer. Overall, the dermis is highly flexible and reliant, but when it is stretched beyond its limits, collagenous and elastic fibres can be torn resulting in "stretch marks" from the repaired scar tissue (Carola *et al.* 1990).

#### 2.1.4.1. Papillary layer

The papillary layer consists of fairly loosely packed connective tissue, with thin bundles of collagenous fibres. Papillae (tiny, finger-like projections) join this layer to the epidermis, through their indigitation with recesses in the epidermis to form dermo-epidermal junctions at their interfaces. Most of these papillae contain loops of capillaries that nourish the epidermis, and others have special nerve endings called corpuscles of touch (Meissner's corpuscles), that serve as sensitive touch receptors. In the glabrous skin, double rows of papillae produce ridges that help to keep the skin from tearing and improve the grip on surfaces. The overlying epidermis follows the corrugated contours of the underlying dermis, and therefore, the double rows of papillae produces distinct fingerprint patterns on the finger pads (Carola *et al.* 1990).

The layer is specialised to provide mechanical anchorage, metabolic support and trophic maintenance to the overlying epidermal tissue. Another function of the layer is to house a rich network of sensory nerve endings and blood vessels (Gray 1987).

#### 2.1.4.2. Reticular layer

This is a netlike layer which is made up of dense connective tissue, with coarse collagenous fibres and fibre bundles that criss-cross to form a strong and elastic network. The collagenous fibres appear to be randomly organised, however, they are arranged so that different directional patterns can be found in each area of the body. The resulting lines of tension over the body are known as cleavage or Langer's lines. The deepest region of the reticular layer contains smooth muscle fibres, especially in the genital and nipple area and at the base of hair follicles (Carola *et al.* 1990)

## 2.2. Skin functions

The skin performs a wide range of protective, perceptive, and regulatory functions. In addition, skin also has good frictional properties, assisting locomotion and manipulation by its texture. Skin's role in providing a protective barrier is, however, most critical for survival. Within limits, the skin is a most effective barrier against microbial invasion and dehydration, and also against mechanical, chemical, osmotic, thermal and photic damage (Gray 1987; Carola *et al.* 2005).

### 2.2.1. Protection from water loss and infection

The skin acts as a stretchable protective barrier that prevents the entry of harmful microorganisms and foreign material into the body. It also functions to prevent water loss from the body. These functions are a result of the layered sheets of epithelial tissue and a nearly waterproof layer of soft keratin in the stratum corneum (Gray 1987; Presland 2009). The skin is, however, not just a passive barrier, but actively defends the body against disease. This is a result of Langerhans cells present in skin (Menon & Kligman 2009).

Langerhans cells can be recognised easily by the presence of Langerhans bodies, which are elongated vacuoles. These cells are regularly scattered throughout the epidermis and are derived from bone marrow cells and continuously renewed. The cell bodies of the Langerhans cells are situated in the base of the stratum spinulosum, whilst their extensively branched dendrites sit between the surrounding cells. Langerhans cells have many features in common with the connective tissue macrophages, including that they are both derived from bone marrow cells and are both thought to be involved in cellular defence, particularly in detecting, binding and presenting antigens to T-cells as part of their immune mechanism in the skin. Such a system appears to be important in cell-mediated immunity to epidermal viral infections, in the elimination of epidermal cancers and many other defensive responses (Asahina & Tamaki 2006). Langerhans cells may also play a role in the regulation of cell division, which is suggested by their closes relation with mitotic epidermal basal cells (Carola *et al.* 1990).

### 2.2.2. Temperature regulation and excretion

The skin can act as a sheet of insulation to retain body heat. To assist in the cooling of the body, dense beds of blood vessels in the dermis dilate to allow heat loss through increased radiation of heat from the blood. To assist in heat retention, these vessels can constrict to

reduce radiation (Gray 1987; Carola *et al.* 1990). Furthermore, since the skin is slightly permeable due to the presence of pores, which are the endings of ducts in the skins surface, skin can also regulate body temperature through the opening and closing of pores and perspiration. These pores allow both the entry of some chemicals and the escape of sweat from sweat glands. The excretion of sweat and its subsequent evaporation causes cooling of the body. Perspiration also allows the excretion of small amounts of waste products such as urea and up to 1 gram of waste nitrogen every hour (Carola *et al.* 1990).

### 2.2.3. Synthesis

Although most of the UV rays are screened out by the skin, it is necessary for the skin to permit the entry of a small amount, which are used to convert the chemical 7-dehydrocholesterol, in the skin, to viamin D3 or cholecalciferol. Vitamin D is essential for the proper growth of bones and teeth. A lack of UV and thus, vitamin D, impairs the absorption of calcium from the intestine into the blood stream (Carola *et al.* 1990).

### 2.2.4. Sensory reception and transduction

Sensory detection is a critical function of skin (Clark *et al,* 2007). The skin contains sensory receptors for heat, pain, cold, touch, and pressure, making it an important sensory organ. The many nerve endings that are present keep us responsive to environmental dangers, and also allow us to make adjustments to maintain homeostasis (Carola *et al.* 1990).

The exact mechanisms of sensory transduction are as yet poorly understood, but some cells involved or thought to be involved in sensory perception have been identified. Merkel cells play a role in sensory transduction. They are only present in glabrous skin, where they lie at the base of the epidermis, usually protruding from there into the dermis. They form the terminal attachment of certain mechanoreceptive cutaneous nerve endings. Merkel cells are elliptical in shape, and from their apical surface several cytoplasmic spikes arise, which insert among the epidermal cells. Hair cells are also known to be involved in cutaneous sense and, keratinocytes are thought to be involved in detection of physical and chemical stimuli (Lumpkin & Caterina 2007).

### 2.2.5. Protection form ultraviolet (UV) radiation

UV protection is provided by melanocytes which produce a dark pigment called melanin. Melanocytes have rounded cell bodies which are usually located in the deepest part of the

stratum basale in contact with the basal lamina, where they extend their long extensively branched dendrites under and around neighbouring cells reaching deep into the stratum spinulosum. Melanin is found in all areas of the skin, but is more concentrated in certain areas such as the armpits and external genitals, and significantly less concentrated in the palms and soles (Carola *et al.* 1990).

Melanin is packaged ino melanosomes and delivered to keratinocytes in the basal layer. Melanosomes are oblong, membrane-bound, rounded bodies which contain the dark pigment, eumelanin. There are also more spherical bodies called phaeomelanosomes which contain a reddish yellow pigment phaeomelanin, and are mostly found in reddish hair. The shape and form of these bodies changes with melanosome maturation Pigment is constantly lost from the epithelium as the basal keratinocytes divide and migrate, and are shed from the skin's surface, so a continuous production of melanosomes is needed (Lin & Fisher 2007).

If too much UV light penetrates the skin, due to inadequate protection, then the radiation may cause acute damage and cell death in epidermal cells, as in sunburn. Alternatively, after chronic exposure, epidermal neoplasms may occur because of damage to the basal cell DNA resulting in squamous cell carcinoma. This occurs because UVB rays can kill some of skin cells and damage others, so that normal secretions are temporarily stopped, increasing the risk of skin cancer and mutations by affecting the genetic material of cells and cause the immune system to falter. UV rays can also damage enzymes and cell membranes and interfere with cell metabolism. If tissue destruction is extensive, then toxic waste products and other resulting debris can enter the blood stream and produce a fever, associated with sun stroke (Carola *et al.* 1990).

The main function of melanin is to screen the harmful UV rays to protect the nucleus and its genetic material in particular. Extra protection is provided when the melanin is darkened by the sun and transferred to the outer layers of the skin, thus causing these layers to become less sensitive to the sun (Carola *et al.* 1990).

Due to genetic differences, darker races have slightly more melanocytes but the differences in the appearance of skin between races are generally reflected by the number and types of melanosomes present. In darker races, more melanin is produced and the melanin is distributed more widely beyond the stratum basale, into the higher strata of the epidermisn.

Various hormones can alter melanization, including melanocyte stimulating homone (MSH), oestrogens and progesterones as well as age and various pathological conditions (Lin & Fisher 2007; Carola *et al.* 1990).

## 2.3. Clinical need for skin replacement

Keratinocytes and fibroblasts present in skin can repair minor wounds caused by trauma or surgery in healthy individuals. If the wound is, however, too large to allow spontaneous healing, or there is an underlying condition which prevents adequate healing, skin graft materials may be used to restore functional integrity and prevent fibrous tissue in-growth. The sections below describe the diseases, both congenital and acquired, and traumas which can necessitate a skin graft.

### 2.3.1. Diseases and congenital skin disorders

A number of diseases exist whereby treatment requires the excision of affected skin or can irreversibly damage skin so that without skin grafting the likelihood of chronic wounds is markedly increased. Some frequently encountered diseases are summarised in this section.

#### 2.3.1.1. Necrolysis

Toxic Epidermal Necrosis (TEN or Lyell's syndrome) and Stevens Johnson Syndrome (SJS) are rare acute dermatological diseases characterised by epidermal necrosis and mucal erosions (Lyell 1956), with extensive loss of contact between the epidermis and dermis and massive keratinocyte apoptosis (Abe *et al.* 2003; Paul *et al.* 1996). More than 10% of the total skin can be lost in TEN, and more than 30% in SJS. The conditions are thought to result mainly from severe adverse drug reaction or, in some cases, bacterial infection (Nassif *et al.* 2004).

#### 2.3.1.2.Graft-Versus-Host Disease (GVHD)

GVHD can develop as a result of allogeneic bone marrow transplantation as a consequence of tissue damage induced by cytotoxic T-cells (Lippens *et al.* 2009). The skin is one of the main organs in which the condition manifests, and large areas of skin can be lost due to apoptosis of keratinocytes (Gilliam *et al.* 1996).

#### 2.3.1.3.Pemphigus diseases

Pemphigus Diseases are autoimmune blistering diseases characterised by the presence of autoantibodies against structural proteins of the intracellular junctions. An example of a pemphigus disease is Pemphigus Vulgaris (Lippens *et al.* 2009). This excessive blistering can result in significant skin loss.

#### 2.3.1.4. Giant congenital epidermal melanocytic nevi

Giant congenital epidermal melanocytic nevi can cover more than 50% total body surface area (TBSA) and can significantly increase the patient's lifetime risk of developing a melanoma if they are left untreated. Melanoma can develop because some of these nevi have the potential to become malignant, particularly if they are exposed to stresses such as excessive abrasion or excessive sunlight (Arneja & Gosain 2009). It is, therefore, often considered desirable to remove the affected skin, thus resulting in the need for a graft.

#### 2.3.1.5. Skin cancer

Epidermal tumours are divided into melanoma and non-melanoma skin cancers, resulting from over-growth of melanocytes or keratinocytes respectively. The most common skin cancers are basal cell carcinomas, which are non-melanoma skin cancers resulting from the overgrowth of basal keratinocytes due to acquisition of mutations. The treatment of tumours associated with skin usually involves complete resection of the affected tissue. Often, this procedure results in the formation of a wound that must be filled by a grafting material (Lippens *et al.* 2009).

#### 2.3.1.6. Epidermal Bullosa (EB)

EB is a group of congenital genetic disorders characterised by erosions and blistering of the epidermis and can result in significant skin loss (Nagy & McGrath 2010; Supp & Boyce 2005).

### 2.3.2. Trauma

Trauma as a result of road-traffic accidents, chemical and thermal burns, or inter-personal violence contributes massively to hospital admissions in the UK, and can all result in significant skin loss. The most common cause of skin loss, however, is thermal injury. Burns can be classified as first, second or third degree, and these classifications are

summarised in Table 2.1. Where burns are third degree, they require a graft (Atiyeh *et al.* 2005; Boucard *et al.* 2007; Mann & Heimbach 1996; Wood *et al.* 2006).

**Table 2.1: Classification of burns.**

| Classification | Description |
|---|---|
| First-degree (superficial) burns | Burns affect only the epidermis, or outer layer of skin. The burn site is red, painful, dry, and with no blisters. Mild sunburn is an example. Long-term tissue damage is rare and usually consists of an increase or decrease in the skin colour. |
| Second-degree (partial thickness) burns | Second-degree burns involve the epidermis and part of the dermis layer of skin. The burn site appears red, blistered, and may be swollen and painful. |
| Third-degree (full thickness) burns | Burns destroy the epidermis and dermis. Third-degree burns may also damage the underlying bones, muscles, and tendons. The burn site appears white or charred. There is no sensation in the area since the nerve endings are destroyed. |

### 2.3.3. Chronic wounds

Chronic wounds do not usually affect a large surface area but are still of high medical and economic impact because they have a high incidence in the general population in the developed world, and generally require grafting to promote healing. Chronic wounds are characterised by excessive and prolonged inflammation and a lack of progression to re-epithelialisation as a result of poor vascularisation of the affected area. They usually occur due to diabetes, pressure and venus stasis (Bannasch *et al.* 2000; Clark *et al.* 2007; Supp & Boyce 2005b). Since chronic wounds do not heal following cleaning and dressing of the wounds, and their incidence is so much higher in the developed world compared with burn injuries, in practise, these wounds are more likely to be treated with TE skin than burn injuries (Kirsner *et al.* 2009).

## 2.4. Wound healing

Wound healing is a complex process involving clot formation, inflammation, re-epthelialisation and tissue remodelling (Martin 1997). The main stages of wound healing are summarised in Table 2.2.

**Table 2.2. The main stages in the wound healing cascade.**

| Stage | Details |
|---|---|
| Fibrin Clot Formation<br><br>Day 0 | Fibrinogen in blood released from damaged blood vessels is cleaved to fibrin by thrombin and a clot forms (Martin 1997). Platelets are embedded in the fibrin mesh, which start to degranulate to release growth factors and cytokines (Babensee *et al.* 1998). |
| Inflammation<br><br>Day 0+ | Neutrophils and monocytes are attracted to the wound by chemoattractants. Neutrophils serve to remove foreign material and bacteria from the wound and express pro-inflammatory cytokines which activate local keratinocytes and fibroblasts. Monocytes become activated macrophages and phagocytose any contaminating microorganisms, cell/ matrix debris and necrotic tissue and express more growth factors and cytokines (Babensee *et al.* 1998). |
| Reepithelialisation<br><br>Day 0+ | Keratinocytes secrete collagenase to allow detachment from the underlying ECM allowing migration over the wound surface from the leading edges of the wound. Keratinocytes behind the actively migrating cells proliferate and subsequently also migrate. Epidermal cells then revert to their normal phenotype and attach to the basement membrane and underlying dermis (Singer & Clark 1999). |
| Wound Contraction<br><br>Day 3+ | Fibroblasts in the dermis assume a myofibroblasts phenotype and draw the wound edges together by connective tissue compaction (Martin 1997). It has also been demonstrated that keratinocytes contribute to the matrix contraction (MacNeil 2007). |
| Granulation Tissue Formation<br>Day 4+ | Fibroblasts are recruited to the wound site and secrete fibronectin-rich ECM, until the space is filled. Blood vessels and macrophages also move into the space (Singer & Clark 1999). |
| Matrix Remodelling<br><br>Day 7+ | Fibrinolytic enzymes such as plasmin are expressed by cells in the clot to facilitate degradation of the clot. Matrix metalloproteins (MMPs) are also expressed by macrophages, epidermal cells, endothelial cells and fibroblasts. MMPs faciliatate cleavage of collagens in the extracellular matrix of both the dermis and the basal membrane (Martin 1997). Cells undergo apoptosis (Desmouliere *et al.* 1995) and the provisional fibronectin-rich ECM is replaced with collagenous matrix. A scar forms (Singer & Clark 1999). |

## 2.5. Conclusion

Skin is a complex structure which performs a variety of functions, most importantly prevention of infection and water loss by provision of a barrier to the external environment. Skin can be lost due to disease, trauma or surgery and due to the complex nature of skin, the healing process is complex. Where wounds do not heal spontaneously, there is a need for technologies to promote wound healing via the wound healing cascade, to restore the barrier function of the skin.

# 3. Skin Repair Technology

In order to promote healing in 'difficult wounds' various approaches have been taken. The gold standard in skin replacement remains the autograft, however, if the affected area is very large, or there is an underlying condition that prevents healing of secondary wounds, alternative strategies must be taken. Other approaches that have been investigated include the use of allografts or xenografts, dressings, growth factors, and tissue engineering. If an approach is to be successful, then the complete structure and function of native skin should be restored.

## 3.1. Skin grafting

### 3.1.1. Autograft skin

An autologous skin graft, or autograft, is harvested from one site in a patient and placed in another. The autograft remains the most frequently used grafting material since it is recognised by the body as self and so does not elicit an adverse immunological reaction. Most frequently, the skin is harvested from the thigh, unless this is the affected area. Disadvantages associated with autograft skin are limited availability, and donor site morbidity and scarring (Atiyeh *et al.* 2005).

### 3.1.2. Allograft skin

Allograft skin is harvested from a member of the same species prior to placement. Use of allograft skin is limited by limited supply the risk of disease transmission, eventual immune rejection and difficulties associated with its storage (Babensee *et al.* 1998; Vuola & Pipping 2002). The immunogenic response to allograft material can be reduced by removing the epidermis, and using only the viable allodermis (Tanner *et al.* 1964). Allogenic skin substitutes act as temporary skin substitutes and can allow early-resurfacing of burn wounds, but allografts often have little effective 'take' (adherence to the wound bed and vascularisation) compared with autografts (Nanchahal *et al.* 2002).

### 3.1.3. Xenograft skin

Xenografts are harvested from one species and implanted into another. The use of most xenograft tissues is excluded because of a vigorous immune response on implantation leading to a high failure rate (Babensee *et al.* 1998; Garfein *et al.* 2003). By removing the epithelium of the skin and fixing it, it is possible to reduce the extent of the immune response, but this

also reduces the bioactivity of the skin. The readily available supply of xenografting materials mean that the problems associated with supply are reduced but concerns over the risk of viral transfer from animal to human (Enoch *et al.* 2005) limit the use of xenografting.

### 3.1.4. Amniotic membranes

Amniotic membranes have been used since 1910 (Rejzek *et al.* 2001). They provide temporary wound coverage, since they cannot become integrated into the host tissue due to their allogenic source. They do, however, allow early healing including epithelialisation, under the amniotic membrane, in superficial wounds but they are not suitable for the treatment of deep burns since the membrane disintegrates faster than epithelialisation occurs (Atiyeh *et al.* 2005).

### 3.1.5. Acellular porcine small intestinal submucosa (SIS)

SIS is an FDA-approved xenogenic scaffold for skin tissue repair. It allows tissue in-growth and degrades *in vivo*. The success of SIS has been attributed to its ECM proteins and glycosaminoglycans, and the cytokines and growth factors that are retained within the matrix (Ratner & Bryant 2004) such as vascular endothelial growth factor (Hodde *et al.* 2001), transforming growth factor $\beta$1 (TGF$\beta$1), and basic fibroblast growth factor (Voytik Harbin *et al.* 1997).

## 3.2. Wound dressings

Traditionally, dressings such as natural or synthetic bandages, cotton wool, lint and gauzes all with varying degrees of absorbency were used for the management of wounds. Their primary function was to keep the wound dry by allowing evaporation of wound exudates and preventing entry of harmful bacteria into the wound. It has now been shown however, that having a warm moist wound environment achieves more rapid and successful wound healing. Modern wound dressings, therefore, are designed to create and maintain a moist environment around the wound and include hydrocolloids, calcium-alginate sponges and hydrogels. Alginate-based dressings include Contreet-H, Seasorb and Algisite M (Paddle-Ledinek *et al.* 2006). The vast array of modern dressings available are reviewed by Paddle-Ledinek *et al.* (2006) and Boateng *et al.* (2008).

### 3.3. Growth factors and cytokines

A variety of growth factors and cytokines are involved in wound healing, including, epidermal growth factor (EGF) (Spiekstra *et al.* 2007), platelet derived growth factor (PDGF) (Greenhalgh 1996), transforming growth factor (TGF-β1) (Puolakkainen *et al.* 1995), insulin-like growth factor (IGF-1), human growth hormone (Steenfos 1994) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Mann *et al.* 2006). Growth factors and cytokines can mediate healing through the stimulation of angiogenesis, cellular proliferation, differentiation and migration, and modification of extracellular matrix metabolism and inflammation (Boateng *et al.* 2008; Gu *et al.* 2004). The effect of localized application of some growth factors and cytokines on wound healing has been investigated.

Direct application of heparin-binding epidermal growth factor (HB-EGF), EGF, GM-CSF, PDGF and keratinocyte growth factor-2/ fibroblast growth factor -10 (KGF-2/ FGF-10) have been seen to accelerate healing (Cribbs *et al.* 1998; da Costa *et al.* 1999; Gaches *et al.* 1998; Jimenez & Rampy 1999; Mann et al. 2006; Marchese *et al.* 2001; Rees *et al.* 1999). The different growth factors have been seen to enhance different aspects of wound healing. KGF-2 has been seen to increase collagen accumulation, strength of wounds and thickness of the epithelium (Jimenez & Rampy 1999), and to induce proliferation and differentiation of human primary cultured keratinocytes (Brown *et al.* 1988; Marchese *et al.* 2001). GM-CSF has been shown to stimulate keratinocyte proliferation, formation of granulation tissue and vascularisation (Mann *et al.* 2001a), and has been used successfully in the treatment of ulcers (da Costa *et al.* 1999; Stagno *et al.* 1999; Voskaridou *et al.* 1999) and amputation wounds (Gaches *et al.* 1998). It has been reported that EGF stimulated epidermal regeneration in partial thickness incisions (Boateng *et al.* 2008; Brown *et al.* 1988).

Other approaches to localized delivery of growth factors have been taken, including injection and controlled release from wound dressings. Basic fibroblast growth factor/ fibroblast growth factor-2 (bFGF/ FGF-2) injected into the dermis of wound margins in full-thickness wounds in humans has been shown reduce scar formation (Ono *et al.* 2007). Dressings which have been seen to enhance wound healing are hydrogel dressing containing transforming growth factor-β1 (TGF-β1) (Puolakkainen *et al.* 1995), collagen film containing human growth hormone (Maeda *et al.* 2001), alginate hydrogel dressing containing vascular endothelial growth factor (VEGF) (Gu *et al.* 2004), and polyurethane and collagen film dressings containing EGF (Grzybowski *et al.* 1999; Park *et al.* 2004).

### 3.4. Tissue engineering

Many skin substitutes have been developed over the last 30 years because the use of autografting is limited. Engineered skin may also be used for *in vitro* applications, to provide a platform for the evaluation of the behavior of new chemical entities in drug and cosmetic development (Faller *et al.* 2002; Faller & Bracher 2002; Hoffmann *et al.* 2003; Hoffmann *et al.* 2005; Khetani & Bhatia 2006; Pu & Bernstein 1995; Souto *et al.* 2006), and as disease models (Jean *et al.* 2009) and aging models (Pageon *et al.* 2008). For grafting purposes, the ideal skin replacement should promote faster and better healing (Clark *et al.* 2007), including enhanced wound closure and reduced scar formation (Nolte *et al.* 2008). At present, no engineered skin substitute can replace all of the functions performed by intact human skin (Ikada 2006; Supp & Boyce 2005).

#### 3.4.1. Epidermal grafts

Since the restoration of barrier function of the skin is often seen as the main priority in wound repair (MacNeil 2007), the first TE skin simply involved the replacement of the epidermis using autologous keratinocytes. This involved the culture and differentiation of autologous keratinocytes *in vitro* to form cultured epithelial autografts (CEA) (Rheinwald & Green 1975b). TE of CEAs was made possible by the establishment of methods for cultivation of keratinocytes *in vitro* with the support of lethally irradiated 3T3 (transformed murine fibroblast) cells in the 1970s (Rheinwald & Green 1975a; Rheinwald & Green 1977), which can allow expansion of enough keratinocytes to culture $2m^2$ of epidermis from a $2cm^2$ biopsy (Pomahac *et al.* 1998). CEAs were first reported in the treatment of major burns in 1981 (Burke *et al.* 1981; Wood *et al.* 2006b), but have been more frequently used in the treatment of chronic wounds (Chern *et al.* 2009). Since the development of the first CEA (Rheinwald & Green 1975b) a number of similar products have been developed, some of which contain pure keratinocyte cultures, and others containing a co-culture of melanocytes and keratinocytes (Green *et al.* 1979; Mak *et al.* 1991; Pu & Bernstein 1995). It has been recognized that although only autologous keratinocytes can be sustained and provide permanent wound coverage, epidermal grafts composed of allogenic keratinocytes can provide temporary wound coverage. Allogenic epidermal grafts serve as biologically active dressings, secreting growth factors and cytokines that enhance wound healing (Phillips & Gilchrest 1991) and is gradually replaced with recipient cells (Gielen *et al.* 1987). Epidermal grafts can be difficult to handle and to address this problem, keratinocyte sprays have been developed. It has been shown that spraying keratinocytes has no adverse effect on cell

viability and that the keratinocytes retain their ability to stratify and form an epidermal layer (Duncan *et al.* 2005; Navarro *et al.* 2000). Epidermal replacements are, however, not ideal skin substitutes as the lack of dermis results in contracture, difficulty in take/ blistering, scar formation and long-term fragility (Donati *et al.* 1992; Kirsner *et al.* 1998; Meana *et al.* 1998a; Still *et al.* 1994; Wood *et al.* 2006b). The effectiveness of epidermal grafts varies, with some studies reporting no appreciable benefit to burn healing (Cuono *et al.* 1986), whilst some studies reported success rates of around 70% in the treatment of chronic wounds (Limat et al. 2003: Poskitt et al. 1987), in terms of restoration of the epidermal barrier layer. Some epidermal replacements which are commercially available or being developed are summarized in Tables 3.1 and 3.2, respectively.

**Table 3.1: Commercially available tissue engineered epidermal replacements.**

| Skin Substitute | Description |
|---|---|
| Epicel | CEA formed from keratinocytes cultured on a petrolatum gauze support (Supp & Boyce 2005; Wright *et al.* 1998). |
| Epidex | CEA formed from keratinocytes isolated from outer root sheath of scalp hair follicles on a silicone membrane support (Supp & Boyce 2005; Tausche *et al.* 2003). |
| Transcell and MySkin | Keratinocyte CEAs on plasma acrylic acid/octa-1,7-diene copolymer supports (Higham *et al.* 2003; Hernon *et al.* 2006; Moustafa *et al.* 2007). |
| CellSpray | Allogenic keratinocyte suspension spray (Navarro *et al.* 2000). |

**Table 3.2: Tissue engineered epidermal replacements being developed.**

| Reference | Description |
|---|---|
| Pu & Bernstein 1995 | Keratinocyte CEA cultured on a nylon mesh microporous membrane support coated in collagen. |
| Horch *et al.* 2000 | Undifferentiated keratinocytes cultured on the surface of a collagen membrane. |
| Horch *et al.* 1998 | Undifferentiated keratinocytes suspended in a fibrin sealant biomatrix. |
| Liu *et al.* 2006a | Autologous keratinocytes cultured on porcine gelatin microcarriers. The microcarriers were first seeded with autologous dermal fibroblasts, which secreted ECM, and are then killed prior to seeding with keratinocytes. |
| Voigt *et al.* 1999 | Collagen-coated dextran microcarriers seeded with keratinocytes. |
| Kamolz *et al.* 2006 | CEA formed from keratinocytes derived from differentiation of umbilical cord blood stem cells cultured on fibrin gels. |
| Wood *et al.* 2007<br>Duncan *et al.* 2005 | Keratinocyte suspension sprays. |

### 3.4.2. Tissue engineered substitutes with a dermal component

The presence of dermis has been shown to support faster re-epithelialisation, better take of epithelial grafts, inhibit wound contraction and improve aesthetic outcome (Kangesu *et al.* 1993; Leigh *et al.* 1993; Navsaria *et al.* 1994).

Most dermal analogues contain a scaffold, which can be used to control and guide wound healing, act as a transplantation vehicle or to provide controlled, localized release of growth factors and cytokines (Babensee *et al.* 1998). Initially, de-epidermalised dermis (DED) and collagen-based scaffolds were investigated as scaffolds for dermal replacement (Bell *et al.*1979; Regnier *et al.* 1981). Since then, collagen scaffolds have been extensively used as analogues of the ECM of the dermis, either in their acellular form or seeded with fibroblasts (Bell *et al.* 1979; Hafemann *et al*. 1999; Helary *et al*. 2010; Powell & Boyce 2006a; Powell *et al.* 2008). Collagen based scaffolds have a number of limitations for tissue engineering skin including excessive contraction (Hafemann *et al.* 1999; Sahota *et al.* 2003), poor vascularisation, blistering and scar formation (Garcia *et al*. 2008; Sahota *et al.* 2004). Poor mechanical properties and rapid degradation rates are the biggest problem associated with collagen scaffolds as this can cause graft instability and difficult handling (Garcia *et al.* 2008). It has been reported that non-crosslinked collagen sponges degrade *in vivo* within three days (Powell & Boyce 2006), and therefore the scaffold degrades much faster than dermal tissue forms. Approaches have been taken to develop collagen-based TE skin matrices with more favorable mechanical properties and degradation characteristics, such as cross-linking with transglutaminase (Garcia *et al.* 2008) or chemicals like EDC (1-ethyl-3-3-dimethylaminopropylcarbodiimide hydrochloride) (Powell & Boyce 2006). Chemical cross-linking of collagen has, however, been shown to reduce chemo-attraction and cellular proliferation and differentiation (Ruszczak 2003).

Collagen scaffolds also present the problem of disease transmission (Alisky 2004) and ethical issues due to their animal source. As a result, other scaffold materials have been investigated for tissue engineering skin, such as fibrin (Cox *et al.* 2004), which can be derived from the patient's own blood, and non-animal derived materials. Fibrin scaffolds, however, also degrade rapidly, and thus may not be the ideal scaffold material for tissue engineering skin. Polyglactin is another material which has been investigated as a scaffold for dermal replacement, and is the material used in Dermagraft. The scaffold has been shown to degrade *in vivo* within 15 days (Cooper *et al*. 1991), which although longer than collagen based

materials, still may be too fast, and may be the reason that although reduced, scar formation and wound contraction still occur with Dermagaft treatment (Hansbrough *et al.* 1993). Furthermore, as dermagraft degrades *in vivo*, the wound pH is altered (Pomahac *et al.* 1998), which may have an adverse effect on keratinocytes and fibroblasts. This may be why when Dermagraft was seeded with fibroblasts and covered with keratinocytes, only half the grafts were seen to be successful in an *in vivo* study (Hansbrough *et al.* 1993).

A number of TE dermal and bi-layer skin replacements are now commercially available, and these are summarized in Tables 3.3 and 3.4 respectively. Since no commercially available product can allow perfect skin regeneration, there is still much research in this area (Ikada 2006; MacNeil 2007; Supp & Boyce 2005). Some of the dermal and bi-layer skin replacements under development are summarized in Tables 3.5 and 3.6, respectively.

**Table 3.3: Commercially available dermal substitutes.**

| Dermal skin Substitute | Description |
|---|---|
| Dermagraft | Neonatal human fibroblasts seeded into bioabsorbable polyglactin mesh scaffold. Extracellular matrix produced in the mesh by the cells (Cooper *et al.* 1991;Marston *et al.* 2003). |
| Alloderm | Acellular human dermis (Wainwright 1995). |
| Graftjacket | Acellular human dermis (Brigido *et al.* 2004). |
| Oasis | Porcine small intestine submucosa (Niezgola *et al*. 2005). |
| SkinTemp | Spongy type I collagen/nylon composite matrix for temporary wound coverage (Kolenik *et al.* 1999). |
| Promogran | Acellular material consisting of 55wt% collagen and 45wt% oxidised regenerated cellulose (Ruszczak 2003;Veves *et al.* 2002). |
| Permacol | Acellular porcine dermis (van der Veen *et al*. 2010). |
| Tiscover | Acellular human dermis with autologous fibroblasts (van der Veen *et al*. 2010). |
| Glyaderm | Acellular human dermis (Pirayesh *et al.* 2008). |

**Table 3.4: Commerically available bi-layer skin analogues.**

| Bi-layer Skin Substitute | Description |
|---|---|
| OrCel | Dermal layer of type I bovine collagen sponge seeded with allogeneic fibroblasts. Allogeneic keratinocytes cultured on the non-macroporous surface of the collagen sponge. Absorbable and permanent (Lipkin *et al.* 2003). |
| BioBrane | Aceullar, flexible cell adherent substrate to be used as a temporary dressing to promote wound coverage. Dermal layer of collagen and nylon covered with a layer of semi-permeable silicone to prevent water loss and allow gas exchange, with an. It has a three year shelf-life (Phillips *et al.* 1994;Tavis *et al.* 1980). Biobrane has been successfully used to treat burns (Purdue *et al.* 1987). |
| TransCyte | A temporary substitute composed of neonatal keratinocytes cultured in a nylon mesh coated with porcine dermal collagen, which is bonded to a silicone membrane (Kumar *et al.* 2004). |
| Integra | Acellular, cell adherant dressing (Kremer *et al.* 2000). Dermal component is a glutaraldehyde cross-linked insoluble bovine collagen and glycosaminoglycan composite. This is covered by a silicone epidermal layer which is removed once the graft has 'taken'. |
| Apligraft (formerly Graftskin) | Dermal component is compised of bovine type I collagen mixed with allogenic dermal fibroblasts. Allogenic keratinocytes are seeded on the upper surface (Falabella *et al.* 1999;Falabella *et al.* 2000;Greenberg *et al.* 2003). The epidermal component is lost either due to chronic rejection or terminal differentiation of all keratinocytes (Mansbridge 2009). |
| Laserskin | A pliable hyaluronic acid-derived dermal matrix seeded (after production) with non-proliferating dermal fibroblasts (feeder layer) and autologous keratinocyctes (Lam *et al.* 1999). |

**Table 3.5: Dermal skin substitutes under development.**

| Reference | Description |
|---|---|
| Fleischmajer *et al.* 1993; Slivka *et al.* 1993 | Human neonatal foreskin fibroblasts grown on a nylon mesh and induced to deposit collagen matrix. |
| Heimbach *et al.* 1988; Yannas & Burke 1980 | Acellular collagen sponge. |
| Chau *et al.* 2005 | Transglutamine cross-linked collagen gel with human dermal fibroblasts seeded on the surface. |
| Chen *et al.* 2000 | Collagen microsponges in the pores of a polylactin mesh seeded with human dermal fibroblasts. |
| Gorodetsky *et al.* 1999 | Fibrin microbeads surface-seeded with fibroblasts. |
| Cox *et al.* 2004 | Human dermal fibroblasts encapsulated in fibrin gel. |
| Garcia *et al.* 2008 | Acellular transglutaminase cross-linked and freeze-dried collagen sponge. |
| Blackwood *et al.* 2008 | Acellular electrospun poly(D,L)-lactide-co-glycolide (PLGA) polymer scaffolds. |
| Sun *et al*. 2005 | Electrospun polystyrene scaffold seeded with human keratinocytes, fibroblasts and endothelial cells. This is a TE skin which could be used for *in vitro* testing, but is not designed for *in vivo* applications due to the non-degradable nature of the scaffold material. |

**Table 3.6: Tissue engineered skin bi-layers under development.**

| Reference | Description |
|---|---|
| Boyce et al. 1995 | Collagen-glycosaminoglycan substrate containing autologous fibroblasts with keratinocytes cultured on the surface. |
| Souto *et al.* 2006 | Human fibroblasts in a bovine collagen type I matrix with a melanocyte/ keratinocyte co-culture on the surface. |
| Powell & Boyce 2006 | EDC cross-linked collagen sponge seeded with human dermal fibroblasts and human keratinocytes. |
| Cuono *et al.* 1986 | DED seeded with cultured keratinocytes |
| Rehder *et al.* 2004 | DED seeded with melanocytes and keratinocytes. |
| Golinski *et al.* 2009 | Matriderm® collagen/ elastin scaffold seeded with fibroblasts and keratinocytes. |
| Lee *et al.* 2006 | ECM scaffold secreted by *in vitro* cultured dermal fibroblasts, surface-seeded with keratinocytes. |
| Kim *et al*. 2005 | Poly(lactide-co-glycolide) biodegradable microspheres seeded with either keratinocytes of fibroblasts for co-transplantation. |
| El Ghalbzouri *et al.* 2004 | Biodegradable co-polymer of PEGT/PBT seeded with fibroblasts to create the dermal layer, then seeded with a CEA cultured on a membrane. |
| Maas-Szabowski *et al.* 2000 | Gamma-irradiated post-mitotic fibroblasts or normal fibroblasts encapsulated in collagen gel and seeded with keratinocytes. |
| Stark *et al.* 1999 | Collagen gel populated with fibroblasts and seeded with keratinocytes. |
| Hafemann *et al.* 1999 | 70% collagen/ 30% elastin porcine membrane dermal analogue seeded with autologous keratinocytes. |
| Meana *et al.* 1998b | Fibroblasts encapsulated in fibrin gel and keratinocytes cultured on the surface. |
| Bannasch *et al.* 2008 | Keratinocytes encapsulated in fibrin and seeded onto Alloderm DED. |
| Boyce 2004 | Dermal component synergistic to Integra seeded with keratinocytes. |

### 3.4.3. Factors affecting clinical uptake of skin substitutes

In order to ensure clinical uptake of a skin substitute it is important to consider the key strengths and limitations of the product. Clinicians often express the desire for an 'off the shelf' product which can be easily stored for extended periods of time until needed (Shevchenko *et al.* 2010). Such products are limited to acellular dermal analogues such as SkinTemp, Permacol, Tiscover, Glyaderm and Alloderm, which are summarised in Table 3.3. Although these products can offer enhanced wound healing, the inclusion of cells has been shown to enhance wound healing by the secretion of growth factors and cytokines (Philips & Gilchrist 1991; Marston *et al.* 2003). Furthermore, where large full thickness wounds are incurred where the hair follicles are lost, there is a need to provide keratinocytes to restore the epidermal layer, since the epidermis cannot be restored by the migration and differentiation of stem cells in the hair follicle (Fuchs 2007), and migration of keratinocytes from the wound margins is often insufficient. In addition, it has been shown that the presence of fibroblasts enhances the formation of a basement membrane (Smola *et al.*1998) increasing the adhesion of the new epidermis to the underlying tissue. This indicates the benefit of inclusion of both keratinocytes and fibroblasts in the production of a skin substitute. Another key consideration in choice of treatment is the associated cost. Cadavaric allograft is one of the cheapest skin substitutes, and is available from not-for-profit skin banks at a cost of around 60p/$cm^2$. Cell-seeded scaffolds are more expensive, due to high amount of skilled labour associated with their production. For example, Transcyte and Dermagraft, which both contain fibroblasts, cost £7.87/$cm^2$ and £7.14/$cm^2$, respectively. Apligraft is approximately twice the pice of Dermagraft and Transcyte, probably dues to the inclusion of both fibroblasts and keratinocytes in this product (Jones *et al.* 2002). The high cost of the material is, however, not necessarily associated with an increased cost of overall treatment. The cost of the overall treatment should also take into account costs associated with outpatient visits, homecare, dressing materials and debridement as well as many other indirect costs, which are often reuced when more expensive skin substiues are used (Langer & Rogowski 2009).

## 3.5. Conclusion

The development of a skin substitute that could perform all the functions of native skin would be beneficial in the clinical setting for the treatment of large and chronic wounds. There are a number of epidermal replacements commercially available and under development, but for the treatment of full thickness wounds, affecting both the epidermis and the dermis, a bi-layer replacement is required for optimal wound healing. The scaffold should degrade at the rate

of new tissue formation and should promote tissue in-growth, vascularisation, innervation and re-epithelialisation. In addition, the scaffold should be from a non-animal source to avoid disease transmission, and should also elicit no adverse immune response.

# 4. Biopolymer hydrogel encapsulation in tissue engineering

There is a current trend in tissue engineering towards the delivery of cells within the material matrix in order to expedite healing (Kretlow *et al.* 2009). Much of this work has involved the use of sponge-like scaffold materials exhibiting interconnected porosity (Rosa *et al.* 2008). Although within such structures, the cells are arranged spatially in three dimensions with respect to one another, they still attach to a two dimensional surface and as such do not exhibit a phenotype that would be expected in native tissue. Since cell phenotype is critical to correct healing of the damaged tissue, a number of workers have attempted to deliver cells within a matrix that is more akin to the extracellular matrix (ECM) than sponge-like polymers. The structure of hydrogels is morphologically similar to that of the ECM of soft tissues (Bokharia *et al.* 2005) due to the high water content and soft consistency of hydrogels (Ratner *et al.* 1976). Hydrogels, therefore enable tencapsulated cell populations to exhibit phenotypes more similar to those *in vivo* than when the cells are grown in monolayer culture (Abbott 2003). Cell encapsulation in biopolymer hydrogels was initially investigated for the treatment of diseases by the sustained release of therapeutic molecules, secreted by the encapsulated cells, and later, for tissue engineering. In this chapter the use of different commonly used biopolymer hydrogels used in cell encapsulation are described, along with the major advantages and disadvantages of each material.

## 4.1. Biopolymer gels

A vast range of different hydrogel based materials are available with contrasting chemistries which may or may not allow cell attachment (Lee & Mooney 2001). A basic overview of a selection of biopolymers utilised for mammalian cell encapsulation in regenerative is given in Table 4.1.

It is essential that materials used in regenerative medicine are able to exist in the body without damaging adjacent cells or leading to significant scarring or otherwise elicit undesirable effects such as excessive inflammation or carcinogenesis (Lee & Mooney 2001). Most biopolymer hydrogels are considered to be suitable for implantation due to the high water content and the minimal mechanical irritation inflicted on surrounding tissue, due to the soft, elastic properties of these hydrogels (Peppas *et al.* 2000). In contrast, synthetic hydrogels frequently display undesirable effects when implanted (Lee & Mooney 2001).

**Table 4.1: Biopolymer gels used for cell encapsulation and tissue regeneration and their key features.**

| Biopolymer | Protein/ Polysachharide | Source | Gelation method | Mechanism of degradation | Cell adhesivity |
|---|---|---|---|---|---|
| Agarose | Polysaccharide | Seaweed | Thermal | Non-degradable | Low |
| Alginate | | | Ionotropic cross-linking | Ion exchange | |
| Carageenan | | | Thermal and ionic cross-linking | Ion exchange and enzymatic degradation | |
| Gellan | | Bacteria | Thermal or ionotropic cross-linking | Thermal or ion exchange | High |
| Chitosan | | Crustaceans and fungi | Ionotrophic or covalent | Ion exchange of stable | |
| Dextran | | Bacteria | Physical, Chemical cross-linking or radical polymerization | Phagocytosis | Low |
| Collagen | Protein | Animal/ human | Neutralisation | Enzymatic cleavage | High |
| Fibrin | | | Proteolytic cleavage | | |
| Gelatin | | | Thermal | | |
| Hyaluron | Polysaccharide | | Chemical cross-linking | | |

### 4.1.1. Alginate

Alginate is a polysaccharide isolated from brown algae which has been used with great success as a wound dressing (Augst *et al.* 2006) and as a food additive. On dissolution in an aqueous medium, monovalent alginate salts form hydrocolloids, which gel ionotropically following the addition of multivalent cations such as $Ca^{2+}$, $Ba^{2+}$ and $Sr^{2+}$. Alginate is formed from polysaccharides derived from a range of seaweeds found world-wide and consists of a mixture of D-mannuronic acid (M) and L-guluronic acid (G) residues (Lee & Mooney 2001). The ratio of M to G blocks can vary significantly depending upon the source of the raw materials used in alginate manufacture. Since hydrogel formation occurs following electrostatic interaction between the carboxylic moieties on the G blocks of alginate and multivalent cations, hydrogel stiffness can be influenced by varying M to G ratios (Wang *et al.* 2003), the properties of main polymer chains/cross-linking molecules (i.e. molecular weight and chain stiffness) (Treloar 1975), and concentrations of the binding cations (Mancini *et al*. 1999). The simplest method for tailoring the mechanical properties exhibited by the alginate, however, is to vary the concentration of alginate in solution (Kong *et al.* 2002). Alginate itself has limited interaction with the majority of mammalian cells due to its hydrophilic character, which promotes limited protein adsorption (Lee & Mooney 2001) and also the lack of cell adhesion ligands in the alginate itself. Cell adhesion to alginate gels can be increased by covalently modifying the polymer with molecules such as the tri-amino peptide, Arginine–glycine–aspartic acid (RGD) (Rowley & Mooney 2002).

### 4.1.2. Fibrin

Fibrin gels occur widely in the human body following injury and are important in haemostasis (Janmey *et al.* 2009). They have, therefore, been used as a sealant and an adhesive in surgery (Lee & Mooney 2001). Fibrin gels form following the cleavage of fibrinogen by thrombin to expose regions on the fibrin molecules that interact allowing self assembly of protofibrils which aggregate and lengthen. At certain points in the fibrin network, branching occurs, which increases the volume of the gel. The presence of RGD motifs within the fibrin network allow cell adhesion and binding of a range of important growth factors. In the presence of mammalian cells, however, fibrin can degrade rapidly due to the localised secretion of proteolytic enzymes (Ye *et al.* 2000). To overcome this problem, fibrin degradation inhibitors and fibrin stabilisers, such as aprotinin, factor XIII and ε-amino-n-caproic acid, have been added to the gels to maintain the structure over longer time periods (Mol *et al.* 2005; Park *et al.* 2005; Ye *et al.* 2000). Furthermore, the importance of sufficient

thrombin and calcium to prevent excessive degradation has been reported (Eyrich *et al.* 2007). A main advantage of fibrin gels is that they can be formed from the patients' own blood so that no inflammatory response would be expected. Also, no toxic degradation products should occur following degradation. The limited mechanical strength of fibrin gels has, however, limited use in clinical applications (Lee & Mooney 2001).

### 4.1.3. Collagen

Collagen is the most abundant form of protein in humans, constituting 30% of all protein found in the body and is the main component of ECMs of skin, bone, cartilage, tendon and ligament (Lee & Mooney 2001). There are twenty nine different forms of collagen found in the body, the most ubiquitous which is type I collagen, which comprises triple α-helices, which in the correct environmental conditions self assemble to form a fibrillar structure (Pachence 1996). The self assembling tendencies of type I collagen have led to it being used as a hydrogel for use in tissue engineering. Rat tail collagen, for example, may be dispersed in acidic medium which, when neutralised in culture, forms gels which have been widely used in regenerative medicine since they allow good cell adhesion. The two major limitations of these collagen based scaffolds are their weak nature and their extensive contraction by encapsulated cells. One group has sought to address both of these drawbacks by plastic compression of the collagen gel immediately after its formation (Brown *et al.* 2005). Alternatively, collagen can be chemically cross-linked using glutaraldehyde (Rault *et al.* 1996) or diphenylphosphoryl azide (Marinucci *et al.* 2003) to improve its physical strength, but this still results in relatively weak gels, and the gels can be potentially immunogenic (Pulapura & Kohn 1992), and toxic due to the release of cross-linking agents with time in culture (Speer *et al.* 1980; Cooke *et al.* 1983). The rapid cell-mediated degradation of collagen gels can also be problematic. To address this problem Chau *et al.* (2005) investigated the use of transglutimase as a cross-linker and Powell and Boyce (2006) have proposed the use of 1-ethyl-3-3-dimethylaminopropylcarbodiimide hydrochloride (EDC) as a cross-linker. Both these methods have been demonstrated to reduce cell-mediated degradation.

### 4.1.4. Gelatin

Gelatin is formed from the hydrolysation of collagen, in which the natural triple helix structure of collagen is broken into single strands (Lee & Mooney 2001). Two different forms of gelatin with different isoelectric points can be formed depending on the

hydrolysation protocol. Gelatin dissolves in water at 60°C then gels as the solution cools to room temperature (Young *et al.* 2005). Due to its ease of manipulation, biodegradable nature and low price, gelatin is widely used in pharmaceutical and medical applications in wound dressings, artificial organs and as a temporary scaffold for damaged tissues (Zandi *et al.* 2007; Ikada and Tabata 1996; Miyoshi *et al.* 2005). As with collagen gels, however, the weakness of gelatin gels has proved problematic in tissue engineering applications (Lee & Mooney 2001). A number of approaches have been taken to improve mechanical properties of gelatin gels such as carbodiimide cross-linking, which has been shown to be successful (Kuijpers *et al.* 1999).

### 4.1.5. Hyaluronic acid

Hyaluronan (HA), a glycosaminoglycan (GAG), is a carbohydrate found throughout the body in the ECM of a number of tissues, including skin and cartilage. HA is a linear polymer of glucuronic acid N-acetylglucosamine disaccharide, which binds cells, either directly through the cell surface receptors CD44, receptor for hyaluronan mediated motility (RHAMM) and intracellular adhesion molecule-1 (ICAM-1), or via cell adhesion to HA-binding proteins known as hyaladherins (Chen & Abatangelo 1999). Colloids of HA can be gelled by prior chemical modification of hyaluronan with thiols, methacrylates or tyramines, or can be cross-linked *in-situ* using formaldehyde or divinyl sulphone. One of the key advantages of using HA gels for tissue engineering is that their degradation can be mediated by hyaluronidase, an enzyme secreted by a multitude of mammalian cell types (Peppas *et al.* 2006). The main disadvantages associated with HA gels are their typically low mechanical properties compared with native tissues and that if the HA is not thoroughly purified, it has the potential to transmit disease, elicit immune response and carry endotoxins (Liu *et al.* 1999). These disadvantages have limited the use of HA gels in tissue engineering applications.

### 4.1.6. Agarose

Agarose is a marine algal polysaccharide which can be formed into a gel that once heated to 90°C forms a polymer solution. When the temperature of this solution is lowered to room temperature gelation will occur, which is reversible upon re-heating (Wong & Mooney 1997). Agarose is used widely in molecular biology and since it is well accepted following implantation it has been evaluated for immunoisolation purposes (Lahooti & Sefton 2000b). The pore size and mechanical properties of agarose gels can easily be tailored by varying agarose concentration (Lee & Mooney 2001). The non-degradable nature of the gel,

however, means that it has not been widely used in tissue engineering since scaffold materials used in tissue engineering applications should degrade over time to allow space for accumulation of new tissue.

### 4.1.7. Dextran

Dextrans are colloidal, hydrophilic, water-soluble polysachharides synthesised by bacteria. Since dextran hydrogels do not affect cell viability, they have been used for many years as carrier systems for a variety of therapeutic agents, including antibiotics, anticancer drugs and peptides (Imren et al. 2010; Hennink *et al.* 1996; Hennink *et al.* 1997; Franssen *et al*. 1997; Franssen *et al.* 1999a; Franssen *et al.* 1999b). Several approaches to preparing dextran hydrogels have been adopted, including chemical cross-linking with N,N'-methylenebisacrylamide, epichlorohydrin or glutaraldehyde (Imren *et al*. 2010) or physically crosslinked by oligo lactic acid stereocomplexes (Bos *et al*. 2005). Generally, dextran hydrogels are seen to degrade very slowly, limiting their applications within tissue engineering. Recently, however, dextran methacrylate esters have been prepared by treating dextran with glycidyl methacrylate (van Dijk-Wolthuis *et al.* 1995). Varying building units and methacrylate offers the possibility to adjust the cross-linking density and may prove an effective method to tailor the degradation of the dextran to the requirements of cell cultivation scaffolds (Moller *et al.* 2007; de Groot *et al.* 2001). Furthermore, although the cell adhesivity of dextran is limited, RGD molecules can be incorporated into the scaffold in order to increase cell adhesion (Ferriera *et al.* 2007; Massia & Stark 2001).

### 4.1.8. Chitosan

Chitosan is an amino polysaccharide (Kumar 2000) prepared by *N*-deacetylation of chitin, the structural element in the exoskeleton of crustaceans (crabs, shrimp, etc.) and cell walls of fungi. Chitosan forms hydrogels by ionic cross-linking (Chenite *et al.* 2000), chemical cross-linking with glutaraldehyde (Mi *et al*. 2000), or by UV irradiation (Ono *et al*. 2000). Chitosan has found many biomedical applications, including tissue engineering, due to its low toxicity, structural similarity to natural glycosaminoglycans, and degradation by enzymes such as chitosanase and lysozyme (Singh & Ray 2000). Since chitosan is positively charged it promotes good mammalian cell adhesion, which is often desirable in tissue engineering applications (Lee & Mooney 2001).

### 4.1.9. Gellan

Gellan gum is a FDA-approved food additive and has been widely exploited in food and pharmaceutical industries (Gong *et al*. 2009). It is a linear extracellular polysaccharide of around 50,000 residues produced by the bacterium *Pseudomonas elodea* (Grasdalen & Smidsrod 1987; Miyamoto *et al*. 2001). Gellan may be considered a preferable choice of scaffold material for tissue engineering because bacterial fermentation can be easilly controlled and offers products of reproducible physical and chemical properties (Abramovic & Klofurator 2006). Both an acetylated and non-acylated form of the polysaccharide are available which form thermoreversible gels. The mechanical properties of the thermally gelled gels can easily be varied by controlling the degree of acylation, from soft and elastic for the acetylated form to hard and brittle for the fully deacetylated polysaccharide (Abramovic & Klofurator 2006), although only the fully deacetylated form is commercially available. Thermal gelation of unmodified gellan, however, occurs above 42°C, thus making it unsuitable for cell encapsulation. It has recently been reported, however, that oxidative cleavage of the gellan backbones can reduce the gelation temperature (Gong *et al*. 2009). Gelation can also occur ionically with both monovalent and divalent cations (Fialho *et al*. 2008; Tang *et al*. 1996). Gellan adsorbs fibronectin (Miyamoto *et al*. 2002), which has specific binding sites for molecules such as heparin and integrin on the cell surface, therefore allowing cell adhesion (Hynes 1990), which is often desirable in tissue engineering applications.

### 4.1.10. Carageenan

There are three forms of carrageenan, known as iota, kappa and lamda, of which the first two can form hydrogels by ionic cross-linking. Gelation of these carrageenans is thought to occur by the simultaneous intra-molecular chain ordering and chain association in the presence of gel-promoting cations (Smidsrod & Grasdalen 1982). Carageenan hydrogels degrade *in vivo*, and there is evidence from studies performed on rats, guinea pigs and monkeys which indicates that degraded carrageenan (poligeenan) may cause ulcerations in the gastro-intestinal tract and gastro-intestinal cancer. Therefore the investigation into the use of carageenan in regenerative medicine has been limited (Tobacman 2001).

## 4.2. Tissue engineering

Cell encapsulation in biopolymer hydrogels was initially investigated for immunoisolation of cells producing therapeutic proteins for treatment of diseases. Some of these studies are

summarised in Table 4.2. More recently, encapsulation of mammalian cells has been used in the regeneration of an array of different tissues. Table 4.3 summarises a broad cross section of the literature covering tissues from the majority of the body.

**Table 4.2: Biopolymer gel cell encapsulation for treatment of diseases with non-autologous cells.**

| Biopolymer Gel | Encapsulated cell type | Secreted therapeutic protein | Disease Treatment | References |
|---|---|---|---|---|
| Agarose | Islet cells | Insulin | Diabetes | Gazda *et al.* 2007; Kobayashi *et al*. 2003 |
| | Kidney cells | Hepatic Lipase | Hyperlipidemia | Lahooti & Sefton 2000a |
| | Fibroblasts | Alkaline phosphatase | Osteoporosis | Lahooti & Sefton 2000b |
| Alginate | Fibroblasts | VEGF | Cardiovascular disease | Keshaw *et al*. 2005 |
| | Kidneys cells | Endostatin | Brain tumour | Joki *et al.* 2001; Read *et al.* 2001a; Read *et al*. 2001b |
| | Myoblasts | Human factor IX | Haeophilia | Hortelano *et al*. 1996 |
| | Fibroblasts | Human factor IX | Haemophilia | Liu *et al*. 1993 |
| | Fibroblasts | Human growth hormone | Dwarfism | Chang *et al*. 1994 |
| | Islet cells | Insulin | Diabetes | Calafiore *et al.* 2006; Soon-Shiong *et al*. 1994; Soon-Shiong 1999; Zimmermann *et al.* 2007 |
| Collagen | Fibroblasts | Alkaline phosphatase | Osteoporosis | Lahooti & Sefton 2000c |

**Table 4.3: A summary of key references from the current literature describing cell encapsulation for the regeneration of a number of tissues.**

| Biopolymer gel | Encapsulated cell type | Engineered tissue | Reference |
|---|---|---|---|
| Agarose | MSCs | Cartilage | Pelaez *et al*. 2009 |
| Alginate | Dorsal root ganglia | Neural | Bellamkonda *et al*. 1995 |
| | Chondrocyctes | Cartilage | Choi *et al*. 2006; Hong *et al*.. 2007 |
| | MSCs | Bone | Smith *et al*. 2007a; Wang *et al*. 2003 |
| | Osteoblasts | Bone | Kong *et al*. 2003 |
| | Hepatocytes | Liver | Khattak *et al*. 2007 |
| | Neural progenitor cells | Neural | Zielinski & Aebischer 1994 |
| Chitosan | Chondrocyctes | Cartilage | Hong *et al*. 2007 |
| | Fibroblasts | Neural | Zielinski & Aebischer 1994 |
| Dextran | ESCs | Blood vessel | Ferreira *et al*. 2007 |
| Collagen | Myofibroblasts | Skeletal muscle | Cheema *et al.* 2003 |
| | Fibroblasts | Dermis | Brown *et al.* 1998; Brown *et al*.. 2005; Chau *et a*l.2005; Nazhat et al. 2006; Marenzana *et al*. 2006 |
| | Fibroblasts | Ligament | Murray *et al*. 2006; Cacou *et al*. 2000 |
| | Fibroblasts | Tendon | Marenzana *et al*. 2006 |
| | Chondrocyctes | Cartilage | Hoshikawa *et al.* 2006 |
| | Osteosarcoma cells | Bone | Bitar *et al*. 2007 |
| Fibrin | Myofibroblasts | Cardiac muscle | Ye *et al*. 2000; Birla *et al*. 2005 |
| | Myofibroblasts | Blood vessel | Mol *et al*. 2005 |
| | Smooth muscle cells | Blood vessel | Grassl *et al*. 2003 |
| | Fibroblasts | Dermis | Tuan *et al*. 1996; Brown *et al*. 1993; Meana *et al.* 1998; Cox *et al*. 2004 |
| | Fibroblasts | Ligament | Chun *et al*. 2003 |
| | Keratinocytes | Epidermis | Bannasch *et al.* 2000; Bannasch *et al.* 2008 |
| | Chondrocytes | Cartilage | Park *et al.* 2005; Eyrich *et al*. 2007; Mesa *et al*. 2006 |
| | MSCs | Bone | Catelas *et al*. 2006; Hou *et al*. 2008 |
| | Hepatocytes | Liver | Bruns *et al.* 2005 |
| | Dorsal root ganglia | Neural | Herbert *et al*. 1998 |
| | Urethral cells | Urethra | Bach *et al.* 2001 |
| | Keratocytes | Corneal stroma | Alaminos *et al.* 2006 |
| Gelatin | Chondrocyctes | Cartilage | Hoshikawa *et al*. 2006 |
| | Hepatocytes | Liver | Wang *et al*. 2006 |
| Gellan | Chondrocytes | Cartilage | Gong *et al*. 2009 |
| | BMSCs | Bone | Smith *et al*. 2007b |
| Hylauronic acid | Chondrocytes | Cartilage | Chung 2008 |
| | Dorsal root ganglia | Neural | Horn *et al*. 2007 |

### 4.2.1. Musculoskeletal tissue engineering

During embryogenesis musculoskeletal tissue develops from the mesoderm. Mesenchymal stem cells (MSCs) differentiate to form the individual musculoskeletal tissues, which include muscle, cartilage, bone, tendons and ligaments. The musculoskeletal system enables locomotion and with the ageing population, novel approaches to regenerate parts of the musculoskeletal system and therefore restore patient mobility are gaining increasing attention.

#### 4.2.1.1. Muscle

In musculogenesis MSCs differentiate to myoblasts, the muscle cell precursors, which further differentiate and fuse to form multinucleated myotubes. These myotubes mature into myofibres which bundle to form skeletal muscle (Berendse *et al.* 2003). Encapsulated skeletal myoblasts have been shown to remain viable and differentiate to form myotubules in collagen gel when mechanical conditioning is used after 7 days culture *in vitro*. Cell alignment was shown to occur uniaxially in the direction of applied tension (Cheema *et al*. 2003).

#### 4.2.1.2. Cartilage

Cartilage is found in joints and prevents bone to bone contact. The tissue consists of mainly chondrocyctes and an ECM containing collagens type I and II, and aggregan (Sterodimas *et al.* 2009). A large amount of research has focussed on tissue engineering cartilage by the encapsulation of chondrocyctes in a variety of hydrogels including fibrin, collagen, chitosan and alginate. Chondrocyctes encapsulated in fibrin gels have been maintained for five weeks *in vitro* (Park *et al.* 2005). Park *et al.* (2005) also showed that addition of HA gel to the fibrin gel may be beneficial since after four weeks *in vivo* culture the degree of contraction was reduced and ECM production was higher in fibrin/ HA gels when compared with pure fibrin gels. Chondrocyctes encapsulated in pure HA gels have been evaluated both *in vivo* up to 12 weeks and *in vitro* up to two weeks post-encapsulation. The constructs were shown to maintain, or increase, in size and encapsulated chondrocyctes were shown to deposit ECM both *in vivo* and *in vitro*. The amount of ECM produced was also shown to be enhanced under mechanical loading (Chung *et al.* 2008).

Chondrocyctes encapsulated in collagen and gelatin gels were shown to produce cartilage specific matrix over 21 days *in vitro* culture. The collagen gel was shown to contract after

one day and an increase in cell number was observed throughout the first 7 days. The viable cell number however, fell gradually over the subsequent 14 days culture. In contrast, the viability of chondrocyctes encapsulated in gelatin hydrogels was maintained, with no significant change in cell number (Hoshikawa *et al.* 2006). Maintaining the viability of encapsulated chondrocytes can be problematic due to insufficient perfusion of scaffolds of large volume. The addition of microchannels to polymeric gels has been proven to maintain the viability and function of alginate encapsulated chondrocytes (Choi *et al.* 2007), and this may prove effective for other polymeric gels.

Recently, the use of low molecular weight gellan to encapsulate chondrocytes by thermal gelation has been investigated. The gels, which were monitored up to 150 days *in vivo*, were shown to retain chondrocyte viability, support extracellular matrix (ECM) secretion and maintain normal chondrocyte phenotype, demonstrating that gellan is a potential and promising material for chondrocyte encapsulation to tissue engineer cartilage (Gong *et al.* 2009).

#### 4.2.1.3. Ligament and tendon

Ligaments and tendons are rich in fibroblasts which secrete ECM containing collagen type I/ III, elastin and proteoglycans (Carvalho *et al.* 2000; Chan and Leong 2008; Cheema *et al.* 2007; Cleary *et al.* 1967). Collagen and fibrin gel encapsulation of fibroblasts have been shown to have promise for tissue engineering of tendons and ligaments. Marenzana *et al.* (2006) showed that tendon fibroblasts encapsulated in collagen gels attached, spread and were orientated in the parallel to the long axis of the collagen fibrils. The fibroblasts produced tensile forces and remodelled the matrix (Marenzana *et al.* 2006). Ligament fibroblasts encapsulated in collagen gels proliferated and secreted further collagen up to day 14, then maintained the collagen level and cell number for a further 7 days culture. Gel contraction was shown to occur over the 21 days culture (Murray *et al*. 2006). Similarly, ligament fibroblasts encapsulated in fibrin gels have been shown to proliferate, contract the gel and secrete ECM (Chun *et al*. 2003). Cacou *et al*. (2000) showed that mechanical loading of collagen gels encapsulating ligament fibroblasts may be beneficial in creation of tissue engineered ligaments, since loading increases the metabolic rate of ligament fibroblasts (Amiel *et al.* 1983) and results in an increase in the gel's structural stiffness after 11 days of cyclic loading.

#### 4.2.1.4. Bone

Bone tissue consists mainly of osteoblast secreted mineralised ECM containing collagen type 1, collagen type IV, fibronectin and heparan sulphate (Narayanan *et al.* 2009; Williams *et al.* 1989). Fibrin gel encapsulation has been investigated as an approach for bone tissue engineering. Where MSCs have been used, their osteogenic differentiation is measured by expression of alkaline phosphatase (ALP), osteopontin, bone sialoprotein (BSP), and osteocalcin. MSCs encapsulated in fibrin gel have been shown to proliferate, but at a slower rate than in monolayer culture. Encapsulation was seen to enhance osteogenic differentiation and ECM production, compared with monolayer culture. Mineralised tissue was seen to accumulate in the pores that formed in the fibrin gel, which were surrounded by numerous cells (Hou *et al.* 2008). Catelas *et al*. (2006) also showed that MSCs encapsulated in fibrin gels proliferate, show osteogenic differentiation, and secrete mineralised tissue. They, however, observed that the MSCs did not fully differentiate to mature osteoblasts within the 28 days of *in vitro* culture. The potential of gellan hydrogels to tissue engineer bone by the encapsulation of bone marrow stromal cells has also been reported. The gellan samples were shown to gel in the presence of the millimolar concentrations of cations present in cell culture media, which allowed for the maintainence of excellent cell viability within the samples. The encapsulated cells were seen to remain viable for at least 21 days encapsulation and appreciable mechanical integrity was maintained throughout this period, although some degradation was observed. This degradation should leave space for new tissue formation, although the synthesis of ECM was not measured (Smith *et al*. 2007b).

### 4.2.2. Skin

For tissue engineering skin fibrin has been used to encapsulate both fibroblasts, to develop a dermal analogue (Cox *et al.* 2004; Meana *et al.* 1998) and keratinocytes to create an epidermal analogue (Bannasch *et al*. 2000; Bannash *et al.* 2008). Encapsulated human neonatal fibroblasts were shown to proliferate within the fibrin matrix, but also allow for the stratification of the co-cultured human neonatal keratinocytes on the surface of the gel both *in vivo* and *in vitro* (Meana *et al.* 1998). Fibrin encapsulated porcine keratinocytes applied to the surface of Alloderm® were shown to produce a continuous epithelium with a cornified layer and basement membrane after four weeks *in vivo* (Bannasch *et al.* 2008).

Brown and co-workers have investigated the use of collagen gels for the encapsulation of human dermal fibroblasts in order to tissue engineer the dermis of skin (Brown *et al.*1998;

Brown *et al.* 2005). Initial work investigated the potential of mechanical loading of these collagen gels to induce matrix contraction and ECM accumulation (Brown *et al.* 1998). Later work, however, focused on the use of plastic compressed collagen gels. The compression was only seen to reduce cell viability of encapsulated human dermal fibroblasts by 10% as long as the gel did not become desiccated (Brown *et al.* 2005). A concern when increasing the collagen content of the hydrogel is that diffusion of nutrients and waste products through the scaffold would not be sufficient to maintain cell viability over extended periods of time. To address this problem Nazhat *et al.* (2007) incorporated micro-channels into the gels using soluble phosphate glass fibres and after 24 hours the cell viability of encapsulated fibroblasts was seen to be greater than 80%.

### 4.2.3. Neural tissue

Chronic neurological diseases and physical injuries can result in loss of neuronal cell bodies, axons and associated glial support. Since the central nervous system has limited or no capacity to replace the lost neurons, there is a significant interest in the engineering of neural tissue (Nisbet *et al.* 2008). One of the major challenges facing researchers in this area is stimulation of guided axonal extension (Norman 2009). To this end, Horn *et al.* (2007) encapsulated chick dorsal ganglia in HA and fibrin gels which were both evaluated *in vitro*. Both gels were shown to support neurites within the first 60h, but after 192h neurite length was seen to be 50% higher in HA gels when compared with the fibrin gels. Despite this, when the neurons encapsulated in the HA gels were implanted, there was no restoration of spinal cord function. Herbert *et al.* (1998) also investigated the use of fibrin gels for neural tissue engineering. Encapsulated chick dorsal root ganglia were successfully maintained in the hydrogels and displayed neurite outgrowth. Bellamkonda *et al.* (1995) showed that PC12 neural progenitor cells and chick dorsal ganglia encapsulated in agarose also produced neurites of 900µm in length after four days in culture.

Another approach to neural regeneration is the localised and sustained release of nerve growth factor (NGF) to stimulate neural differentiation of precursor cells. For example, Zielinski & Aebischer (1994) have shown that a genetically modified fibroblast cell line provided sustained release of NGF during encapsulation in chitosan hydrogels. The amount of NGF secreted was sufficient to induce the differentiation of co-cultured neural progenitor PC12 cells. The limitation of this approach, however, was that fibroblasts were seen to

aggregate during the course of the study and after two weeks which resulted in necrosis in the centre of the aggregates.

### 4.2.4. Liver

Hepatic tissue is formed of hepatocytes which are organized into a polarized epithelium with distinct apical and basal domains (Dunn *et al.* 1989). Hepatocytes have a high metabolic activity and produce many liver specific molecules such as urea, amino acids, glycogen and bile. The successful tissue engineering of liver is often assessed by measuring the secretion of these liver specific products.

Hepatocytes encapsulated in fibrin maintained viability over the first three days culture *in vivo*, but thereafter showed significant loss despite the maintenance of an even distribution of cells. Neotissue formation, however, was observed with good integration into the host tissue after 7 days *in vivo* as well as maintained liver specific function and phenotype in viable cells (Bruns *et al*. 2005). In order to maintain encapsulating hepatocytes viability and function various approaches have been taken to facilitate the diffusion of nutrients within hydrogels. For example, the incorporation of micro-channels into gelatin hydrogels was shown to maintain 90% viability and metabolic activity of the encapsulated hepatocytes after 45 days *in vitro* culture (Wang *et al*. 2006). Similarly Khattak *et al*. (2007) have shown that perfluorocarbon incorporation improved metabolic activity and viability of encapsulated hepatocytes cultured *in vitro* for two weeks.

### 4.2.5. Cardiovascular tissue

The cardiovascular network is essential to maintaining the viability and functions of all living tissues. The network can become obstructed of damaged due to disease or injury and thus efforts towards the tissue engineering of blood vessels and cardiac tissue are being made (Stegemann *et al*. 2007). In order to engineer vascular tissue, myofibroblasts have been encapsulated in fibrin gels (Mol *et al*. 2005; Ye *et al*. 2000) and viability maintained for up to six weeks *in vitro* and the cells were also seen to proliferate and secrete collagen. Smooth muscle cells have also been encapsulated in fibrin gels in order to produce an arterial media equivalent. After three weeks *in vitro* culture the arterial equivalent was seen to have a 95% reduction in volume and was shown to accumulate collagen, which was aligned in the circumferential direction of the tubular structure. The accumulation of collagen and the gel

contraction was associated with an increase in ultimate tensile strength of the construct (Grassl *et al*. 2003).

Dextran hydrogel has also been investigated as a scaffold for tissue engineering blood vessels. Ferriera *et al*. (2007) showed that human embryonic stem cells (ESCs) encapsulated in photocrosslinked dextran-methacrylate hydrogels survived for at least 10 days *in vitro*. The cells were encapsulated as aggregates as this was seen to promote better cell viability compared with monodispersed cells. They found that the encapsulated cells displayed enhanced vascular differentiation compared to embyoid bodies formed in 2D culture. The encapsulated cells, as compared with monolayers, were reported to have increased expression of the VEGF receptor, KDF, a marker of vascular differentiation, and reduced expression of markers of differentiation to other undesired cell types such as hepatocytes and neurons. During the study, however, no blood vessels were actually formed within the hydrogel, perhaps due to study being only 10 days long.

Cardiac tissue has successfully been engineered *in vivo* by encapsulation of neonatal cardiac myocytes in fibrin gel within a silicon chamber implanted near an artery to maintain cell viability. After three weeks the implant was found to be viable with large amounts of muscle tissue which contracted in response to electrical stimulation and had neovascularisation throughout (Birla *et al*. 2005).

### 4.2.6. Urethral tissue

The urethra is a tube which connects the urinary bladder to the outside of the body. The tissue consists of an epithelial layer lining the lumen of the tube, and a thicker muscular layer. Damage or loss of the urethra can occur due to congenital defects, acquired disorders and injury. Since only a small quantity of genitourinary tissue or mucosa is available for grafting, and other sources of tissue often show significant complications, there has been an interest in tissue engineering of the urethra (Magnan *et al*. 2009).

Bach *et al*. (2001) have investigated the encapsulation of primary autologous urothelial cells in fibrin gel for the tissue engineering urethral tissue. The encapsulated cells were applied to the surface of a pre-fabricated capsule tube *in vivo* and after 14 days histological and immuno-staining revealed that viable epithelial tissue similar to the native tissue had formed. The cells were reported to have proliferated, orientated appropriately, adhered and become

confluent. In addition, the fibrin was reported to have fully degraded and been replaced by connective tissue. After 28 days *in vivo* dense fibrous tissue was seen to have formed and vascular proliferation was evident as well as a mild inflammatory response. This indicates that fibrin encapsulation of urothelial cells may be suitable for tissue engineering the urethra. It is possible that cell encapsulation in other biopolymers may also be suitable, but at present there appears to be limited research in this area.

### 4.3. Conclusion

The most widely used biopolymeric hydrogels in tissue regeneration have been summarised. Researchers have studied a variety of factors including cell viability, growth, matrix production and differentiation. It is clear is that alginate, collagen, fibrin, gellan, chitosan, hyaluronic acid, and gelatin all show promise as materials for the encapsulation of cells for tissue engineering. Alginate in particular shows great potential for the encapsulation of mammalian cells in regenerative medicine due to its ready availability, low cost, low immunogeneity, FDA-approval for medical applications, non-animal source, ease of gelation, relatively slow degradation, and proven support of cell viability and function of a variety of encapsulated cells. Alginate hydrogel was, therefore, chosen as the scaffold material for the encapsulation of fibroblasts in order to produce the dermal layer of the tissue engineered skin.

# 5. Methods and materials

## 5.1. Fibroblasts

NIH 3T3 murine fibroblasts were obtained from LGC (Middlesex, UK).

### 5.1.1. Fibroblast culture

FCM was prepared by supplementing high glucose Dulbecco's modified Eagle's medium (DMEM) (Sigma, UK) with 10% v/v foetal bovine serum (FBS) (PAA, Somerset, UK), 1% v/v penicillin-streptomycin (P/S), 2.25% v/v 1M 4-(2-hydroxyethyl)-1 piperazineethane-sulfonic acid (HEPES) and 2% v/v L-glutamine. All cultures were maintained at 37ºC with 5% $CO_2$ and 100% relative humidity and media was changed three times weekly.

## 5.2. Alginate hydrogel preparation

Alginate hydrocolloid solutions of either 2% or 5% w/v concentration were made by gradually dissolving low viscosity (20-40 centipoise (cps) for 2% w/v at 25ºC, (Sigma UK, Cat number 180947, lot 08620BJ, MW 102000-209000, M:G ratio 1.56) sodium-alginate in double distilled water on a heated stirrer plate. All hydrocolloids and other solutions required for gel preparation were sterilised by autoclaving for 2h at 121°C and 1bar.

NIH 3T3 cells (LGC, Middlesex, UK), passaged at least twice after storage in liquid nitrogen and pelleted by centrifugation at 167 x g (1000rpm) for 3min, were suspended at a density of $7.5x10^5$ cells/ml of alginate hydrocolloid. The alginate/3T3 dispersion or acellular alginate hydrocolloid was added dropwise into a bath of 100mM $CaCl_2$ (Sigma, UK) and left to incubate at 37ºC for 2h to form cross linked spheres of 3.0±0.2 mm diameter. To form alginate discs, the hydrocolloids were used to cover the surface of a petridish, on which silicone disc shaped molds were placed, and immersed in 100mM $CaCl_2$ for 3h to allow for complete cross-linking of the alginate. The displacement of alginate by calcium chloride was prevented by covering the alginate hydrocolloid with a layer of filter paper impregnated with 100mM $CaCl_2$. After incubation in $CaCl_2$ the samples were washed three times in DMEM before culture in FCM. Cells were released from alginate hydrogel by incubation of the gel in 100mM tri-sodium citrate (Sigma, UK) solution for 2h at 37ºC.

Alginic acid is a linear polymer composed of α-L-guluronic acid (G) and β-D-mannuronic acid (M) units in varying proportions and sequential arrangements. Calcium cations are

thought to electrostatically bind with the alginate, preferentially to the poly-guluronic acid units (GG) in a planar two-dimensional manner, producing the so-called "egg-box" structure, where the negatively-charged carboxyl groups are accessible due to the epimerization of the fifth carbon (Park *et al*. 1993).

### 5.3. Live/ dead staining

Samples were immersed in 0.2µg/ml Calcein acetoxymethylester (Calcein-AM) for 15min and 2.5µg/ml Propidium Iodide (PI) (Invitrogen, Paisley, UK) for 5min in supplemented DMEM at 37ºC to stain live cells green and dead cells red, respectively, when visualised at 490nm using fluorescence microscopy with a mercury light source. Propdium Iodide and Calcein-AM were stored at -20°C until required. PI was diluted in water to a concentration of 100ug/ml and Calcein-AM was diluted in DMSO to a concentration of 50µg/ml to form stock solutions which were stored at 4°C in darkness. Calcein-AM is highly lipophilic and cell membrane permeable. Calcein-AM itself is not a fluorescent molecule, but the calcein generated from Calcein-AM by esterases in the cytoplasm of viable cells emits strong green fluorescence, and therefore, Calcein-AM only stains viable cells. PI is a nuclei staining dye which cannot pass through a viable cell membrane. It reaches the nucleus by passing through disordered areas of dead cell membrane, and intercalates with the DNA double helix of the cell to emit red fluorescence and therefore, only stains dead cells. Since both calcein and PI-DNA can be excited at 490nm, simultaneous monitoring of viable and dead cells is possible with a fluorescence microscope. The excitation maxima and emission of calcein and DNA-PI are 495nm and 535nm, respectively.

### 5.4. Trypan blue exclusion/ haemocytometer counts

In order to ascertain cell number using an improved Neubauer haemocytometer (Beckman Coulter Ltd, UK), cells were centrifuged at 167 x g (1000rpm) for 3min and resuspended in a small volume of culture medium then mixed 1:1 with a 0.2% w/v solution of trypan blue in phosphate buffered saline (PBS, NaCl 138mM; KCl 27mM, pH 7.4 at 25 °C, Sigma, UK). The Haemocytometer was then viewed under a light microscope and the number of blue (dead) and non-stained (viable) cells counted and used to ascertain the viable cell number according to the method described by Butler (2004). The method is based on the principle that live cells possess intact membranes that exclude trypan blue, whereas dead cells do not, and therefore viable cells will have a clear cytoplasm whereas non-viable cells will have a blue cytoplasm.

### 5.5. Thiazolyl blue (MTT) assay

3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) is a yellow water-soluble molecule which readily passes through cell membranes and into the mitochondria. MTT is reduced to water-insoluble blue crystals of formazan by mitochondrial succinate dehydrogenase. When cells are solubilised with isopropanol the formazan is released and solubilised.

#### 5.5.1. MTT assay for growth

A stock solution of sterile-filtered 5mg/ml MTT (Sigma, UK) in phosphate buffered saline was prepared and stored at 4°C in darkness. The MTT stock was added to supplemented DMEM at a concentration of 10% v/v. 600µl samples of alginate/ 3T3 beads were removed from the media, carefully washed in DMEM and immersed in the resulting 10% solution of MTT for 18h. Monolayer cultures of fibroblasts were plated at a density of $4.4\times10^5$ cells/ well in 6 well plates. These monolayers were either 1) with no pre-treatment, 2) treated with 100mM calcium chloride for 2h at 37ºC, 3) treated with 100mM tri-sodium citrate for 2h at 37ºC, 4) in the presence of a piece of alginate suspended above the cell monolayer in a Thincert TM well insert (Greiner, Stonehouse, UK) or 5) released from an alginate hydrogel by immersion of the alginate hydrogel in 100mM tri-sodium citrate for 2h at 37ºC. The MTT solution was then removed and replaced with 100mM HCl in isopropanol to lyse the cells and left for 2.5h at 37°C to allow the formazan produced to dissolve and diffuse out of the alginate. The optical absorbance of the solution at 620nm was measured using a spectrophotometer (Cecil, Cambridge, UK). The amount of formazan produced by cellular reduction of MTT was determined from the absorbance at 620nm according to a calibration of different cell concentrations in alginate or in monolayer culture as determined by counting using a haemocytometer and the absorbance at 620nm after application of the MTT assay.

#### 5.5.2. MTT assay for mitochondrial activity

500µl samples of alginate/ 3T3 beads or $3.75\times10^5$ monolayer cultured fibroblasts/ well in 6 well plates were immersed in 10% MTT reagent (v/v) and the amount of formazan produced was assessed by spectrophotometer as described above. Measurements were made hourly so that the metabolic activity of fibroblasts under different conditions could be compared.

### 5.6. Measuring mitochondrial membrane potential

It has been suggested that the ability of 5,5',6,6'-tetrachloro-1,1',3,3'tetra-ethylbenzimidazolylcarbocyanine iodide (JC-1) to probe the membrane potential depends on the reversible formation of aggregates (Reers *et al.* 1995). The JC-1 monomers, which fluoresce green, should accumulate within the aqueous matrix space resulting in formation of aggragates, which fluoresce red, once the critical concentration of monomers has been reached (Reers *et al*. 1991). De-energising conditions such as metabolic inhibition applied to the cells reduce the aggregate emission and increase the monomer emission (Dilisa *et al*. 1995).

Encapsulated or monolayer cultures of 3T3 fibroblasts were immersed in FCM containing 1µm/ml JC-1 (Invitrogen, UK). The samples were then incubated at 37ºC in the dark for 15min. The green and red fluorescence at 490nm were visualized using a fluorescence microscope with a mercury lamp. The amount of green and red fluorescence seen in different samples after 15min was used as an indicator of metabolic activity under different conditions.

### 5.7. Determining alginate degradation by change in mass

500µl samples of alginate beads were prepared and immersed in 4ml of FCM. The alginate hydrogel masses were assessed at various time points after *in vitro* culture. Masses were measured before and after degradation in a vacuum freeze-drier (Edwards, EF03, Sussex, UK) for 16h. 500µl samples were removed from culture, blotted carefully on paper towels to remove excess surface moisture then weighed on a top pan balance. Samples were measured in triplicate and the mean and standard deviation for each data point were shown.

### 5.8. Inductively coupled plasma - mass spectroscopy (ICP-MS)

1ml samples of alginate beads were prepared and immersed in 4ml of FCM. Media was changed three times weekly then left unchanged for four days prior to removal of media for analysis of calcium, phosphate, potassium and sodium ion content using ICP-MS (Varian, Darmstadt, Germany) against standard solutions of 50ppm and 100ppm (Merck, Darmstadt, Germany). Samples were performed in triplicate and mean values±SD shown.

The sample is pumped into a nebulizer, where it is converted into a fine aerosol with argon gas. The fine droplets of the aerosol are separated from larger droplets using a spray chamber. The fine aerosol then emerges from the exit tube of the spray chamber and is transported into

the plasma torch via a sample injector, where the gas is ionized. When the ionized gas is seeded with a source of electrons from a high-voltage spark, it forms a plasma discharge with a temperature of 500-800K. Once the ions are produced in the plasma, they and directed into the mass spectrometer and electrostatically focused to the detector. Analyte ions of a particular mass-to-charge ratio (m/z) are allowed through to the detector whilst all the non-analyte ions are filtered out. The ions are sampled and an ion detector converts the ions into an electrical signal. This electronic signal is then processed by the data handling system in the conventional way and converted into analyte concentration using ICP-MS calibration standards (Becker 2007).

### 5.9. Rheology

The word 'Rheology' comes from the Greek word 'Rheo', meaning to flow, and can be defined as the relationship between stress and strain within a material as a function of time, temperature, frequency etc. Stress is the force per unit area acting on a sample and is measured in Pascals (Pa). Strain is the resulting fractional deformation and therefore is a dimensionless ratio.

According to their mechanical properties, materials can superficially be classified as either solids or liquids. These two extremes of behavior correspond to a perfect (Hookean) solid or a perfect (Newtonian) liquid. In Hookean solids, such as a steel rod, stress ($\sigma$) is proportional to strain ($\gamma$) and independant of the rate of strain or oscillatory frequency (Clark & Ross-Murphy 2009). For Newtonian liquids, stress is proportional to the rate of strain but independent of strain itself. Most materials, including gels, are referred to as viscoelastic materials meaning they display some solid (elastic) behaviour and some liquid (viscous behaviour).

#### 5.9.1. Small deformation oscillatory rheology

Most modern rheometers employ oscillatory shear, with controlled stress. In the simplest form, a small sinusoidal strain wave is applied to the top surface of a gel and the resultant stress transmitted through the sample is measured. In general, the stress and strain waves vary in both phase and amplitude, but using phase resolution the in-phase and 90° out-of-phase components can be extracted. $G'$ is the storage or elastic modulus given as a ratio of in-phase stress divided by strain, and $G''$ is the loss modulus, the ratio of out-of phase stress to strain (Clark & Ross-Murphy 2009). $G'$ is a measure of the energy stored in the material

and indicates solid or elastic behaviour. G″ characterises the liquid or viscous behaviour (Zandi *et al.* 2007). Combination of both parameters provides important information on viscoelastic behaviour of polymer gels. Viscoelastic solids have a finite G′, with a value usually well above (5-50×) that of the G″, at all frequencies. G′ and G″ may also show an apparent increase with strain, although this is more difficult to generalise (Clark & Ross-Murphy 2009).

### 5.9.2. Mechanical spectroscopy

From the G′ and G″ values the complex viscosity, can be calculated as follows:
Firstly, the complex modulus, G*, is given by:

$$G^* = \sqrt{(G')^2 + (G'')^2} \quad (1)$$

And the ratio:

$$\frac{G''}{G'} = \tan(\delta) \text{ where } \delta = \text{the phase angle} \quad (2)$$

And finally, the comple viscosity, η*, is given by:

$$\eta^* = \frac{G^*}{\omega} \text{ where } \omega = \text{the oscillatory shear frequency given by } 2\pi \times \text{frequency in Hertz.} \quad (3)$$

### 5.9.3. Monitoring gel degradation by small deformation oscillatory rheology

The degradation of 2% and 5% w/v acellular and fibroblast encapsulating alginate hydrogel discs of 20mm diameter and 5mm height were monitored by capturing images of the discs after 28 days *in vitro* culture (Canon Powershot GS, Surrey, UK) and by performing small deformation oscillatory rheology at days 1, 7, 14, 21 and 28. For both 2% and 5% w/v alginate hydrogels the effect of cell culture media on alginate degradation was assessed by comparing acellular samples incubated in FCM with acellular samples incubated in water. The effect of encapsulated cells on degradation was assessed by comparing acellular samples and cell-encapsulating samples incubated in culture media. All cultures were maintained at 37ºC with 5% $CO_2$ and 100% relative humidity and media or water was changed three times weekly. Samples were trimmed to size where necessary using a razor blade to obtain samples

of 20mm diameter with planar surfaces. Measurements were taken in the linear viscoelastic region using parallel plate geometry 20mm mounted on a Bohlin CVO Rheometer (Malvern Instruments, UK) fitted with Peltier plate thermal control. The plate gap corresponded to sample thickness. Measurements of storage modulus (G′), loss modulus (G″) and dynamic viscosity (η*) were taken at frequencies from 1rad/s to 100rad/s to ascertain mechanical spectra of the gels at an isotherm of 37°C and at a fixed strain of 0.5%. Measurements were performed in triplicate and mean values shown. Comparisons of G′, G″ and η* were made at a frequency of 6rad/s.

### 5.10. Keratinocytes

Primary keratinocytes were isolated from neonatal rats. Primary cultures were established by sacrificing one to two day old neonatal rats by cervical dislocation. The rats were kept on ice prior to tissue dissection to minimise tissue deterioration. After cleaning by bathing in a concentrated solution of iodine dissolved in 70% ethanol followed by washing in 70% ethanol and then sterile double distilled water, the bodies were placed on a polystyrene board and immobilised by needles through the extremities of the limbs. The skin was then cut away using dissection scissors and excess fatty tissue and dermis cut away before placing the skin dermis-down on 0.25% trypsin-0.1% EDTA (ethylenediaminetetraacetic acid). The skin was left for 18h at 4°C on the trypsin-EDTA then using forceps the epidermis was mechanically separated from the dermis. The epidermal layer was then minced and placed in FCM with a magnetic stirrer bar and placed upon a stirrer plate at approx. 100rpm (16.7 x g) for 30min at 4ºC. The stirring releases cells from the epidermal sheet. The suspension was then strained through a tea-strainer so that released cells passed through and the cornified layers of the epidermis did not. The cell suspension was then seeded onto fibroblast feeder layers at a density of approx. $3\text{-}5\times10^5$ cells/ T75.

#### 5.10.1. Keratinocyte culture

Keratinocyte culture medium (KCM) consisting of 3:1 DMEM:Ham's F12 (Sigma, UK) supplemented with 50mM hydrocortisone, 10µg/ml insulin, 25ng/ml EGF, 10% v/v FBS (PAA), 1% v/v P/S, 2.25% v/v HEPES and 2% v/v L-glutamine was used for the expansion of keratinocyte cultures.

To discourage primary fibroblast over-growth, the media was removed and replaced with 0.02% w/v EDTA in PBS. The cells were then incubated for 30sec at 37°C and aspirated vigorously to detach fibroblasts and leave the keratinocytes attached.

3T3 feeder layers were prepared by incubating 3T3 monolayers in a 4µg/ml solution of Mitomycin C (Sigma, UK) in FCM. The solution was removed, then cells were washed three times in PBS before being trypsinised in trypsin-EDTA for approx 3-5min at 37°C, centrifuged at 1000rpm (167 x g) for 3min, counted and used for support of keratinocyte cultures. Approx. $1\times10^6$ feeder cells were seeded into T75 flasks for use as feeder layers.

### 5.10.2. Epidermal analogue culture

In order to tissue engineer epidermal analogues keratinocytes were seeded onto the surface of either alginate/ 3T3 dermal analogues or Thincert TM 6 well plate inserts with pores of 0.4µm diameter (Greiner Bio One, UK) at a density of $2\times10^5$ cells/cm$^2$. Where Thincerts were used, a 3T3 feeder layer was present on the base of the multiwall dish below. The keratinocytes were cultured for 7 days in KCM on these surfaces before being raised to the air-liquid (A/L) interface and cultured in 3:1 DMEM:Ham's F12 supplemented with 10% FBS (PAA), 1% v/v P/S, 2.25% v/v HEPES and 2% v/v L-glutamine to allow for stratification of the keratinocytes. All keratinocyte cultures were maintained at 37ºC with 5% $CO_2$ and 100% relative humidity.

## 5.11. Endothelial cells (ECs)

Immortalised mouse coronary ECs were a gift from Prof. Gerard Nash (University of Birmingham Medical School). Endothelial cell culture medium (ECCM) was composed of Medium 199 with L-glutamine (Invitrogen, UK) supplemented with 20% foetal bovine serum (PAA, UK), 1µg/ml mouse hydrocortisone, 10ng/ml mouse EGF, 1% v/v p/s, 1% v/v Fungizone (Gibco, UK) and 2.25% v/v HEPES. EC cultures were maintained at 37ºC with 5% $CO_2$ and 100% relative humidity and media was changed three times weekly.

### 5.11.1. EC migration assay

The ability of the dermal analogue to induce migration of ECs was investigated. $7.5\times10^5$ ECs were plated onto 6 well tissue culture (TC) treated transwell support with 8µm diameter pores (Fisher Scientific, UK). These supports were placed in deep-well 6 well plates which were seeded with 7ml of 2% or 5% w/v fibroblast encapsulating/ acellular alginate to which 14ml

ECCM was added. This medium was left unchanged throughout the course of the study. At different time points, EC cells present on the underside of the transwell supports (which had migrated through the pores in the transwell supports) were were removed with a cell scraper and suspended in PBS. The number of cells was then determined by counting on a haemocytometer. Samples were performed in triplicate and mean±SD values are shown.

### 5.11.2. EC proliferation assay

The ability of the dermal analogue to induce proliferation of ECs was investigated. $2.5\times10^5$ ECs were plated onto 6 well TC treated transwell support (Fisher Scientific, UK) with 0.4µm diameter pores. These supports were placed in 6 well plates which were seeded with of 2% or 5% w/v fibroblast encapsulating/ acellular alginate, as for the EC migration experiments. This media was left unchanged throughout the course of the study. At different time points, the number of EC cells present was determined by counting on a haemocytometer, after trypsinisation in trypsin-EDTA fro approx. 3-5min at 37°C. Samples were cultured in ECCM which was changed three times weekly. Measurements were performed in triplicate and mean±SD values are shown.

## 5.12. VEGF enzyme-linked immunosorbent assay (ELISA)

ELISA is an immunoassay technique involving the reaction of antigen and antibody *in vitro*. ELISA is a sensitive and specific assay for the detection and quantitation of antigens or antibodies. The ELISA kit used employs the quantitative sandwich enzyme immunoassay technique. A polyclonal antibody specific for mouse VEGF is pre-coated onto a microplate. Any mouse VEGF present is bound by the immobilized antibody and after washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse VEGF is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The intensity of the colour measured is in proportion to the amount of mouse VEGF bound in the initial step. The VEGF concentrations are then read off the standard curve according to the absorbance at 450nm.

The amount of VEGF secreted by 3T3 fibroblast monolyers and 3T3s encapsulated in 2% and 5% w/v alginate hydrogels was determined by VEGF ELISA (R&D Research Systems, Abingdon, UK). 3T3 monolayers were seeded at a density of $4.4\times10^5$/ well of a 6 well plate, and 1ml samples of alginate containing $7.5\times10^5$/ml were used. Samples were cultured in 4ml

FCM which was changed three times weekly and left unchanged for three days before sampling. Media samples were collected and stored at -20°C for up to four weeks before assaying, in accordance with the manufacturer's instructions. Measurements were performed in triplicate and mean±SD values are shown.

### 5.13. Subculture and freezing

Phosphate buffered saline (PBS) was obtained for Sigma (UK) and used to wash monolayer cultures. 0.25% w/v trypsin/ 0.1% EDTA was obtained from Sigma (UK) and used for detachment of cultured cells on tissue culture flasks to prepare cell suspensions for sub-culture or freezing. 10% v/v DMSO (dimethyl sulfoxide) in FCM or KCM was used to protect cells during frozen storage, by preventing the formation of ice crystals. Fibroblasts and ECs were split 1:6 and keratinocytes 1:2 for passaging. All cells were generally frozen at a density of $1\times10^6$ cells/ml. Cell densities for seeding were measured using a haemocytometer.

### 5.14. Histology and immunostaining

#### 5.14.1. Histological Stains

Once tissue sections or cells are firmly attached to glass microscope slides, various staining methodologies can be applied to visualize cell and tissue structure and composition. Visualization is facilitated by the application of dyes, the deposition of heavy metals and the use of chemical reactions

The staining procedure most commonly used is the haematoxylin and eosin (H&E) method which demonstrates the general architecture of tissue. The haematoxylin stains the cell nuclei and other negatively charged structures such as alginate blue-black while the eosin stains cell cytoplasm and most connective tissue fibres in varying shades of pink, orange and red (Gamble & Wilson 2002). H&E staining was performed using a Shandon Linistain GLX Random Access Stainer (Thermo Scientific, UK). Samples were heated to 60°C for 4.5min, then immersed in xylene for 1min, IMS 99 (Genta Medical, UK) for 40sec, 95% IMS 99 for 40sec, rinsed in tap water for 20sec, immersed in Gill's III Haematoxylin (Surgipath Europe Ltd, UK) for 2.5min, rinsed in tap water for 20sec, immersed in 0.3% w/v acetic acid (Merck Ltd, UK) in distilled water for 20sec, 0.3% HCl (Genta Medical, UK) in 70% IMS 99, rinsed in water for 20sec, immersed in Scott's tap water substitute (Surgipath Europe Ltd, UK) for 40sec, washed in tap water for 20sec, immersed in Eosin (Surgipath Europe Ltd, UK) diluted

1:1 with 70% IMS 199 for 20sec, rinsed in tap water for 20sec, immersed in IMS 199 for 1min and zylene for 50sec. Following this the samples were held in xylene for up to 4h before application of cover slips.

Collagens are the most abundant component of most tissue ECM including the dermis of skin. Collagens stain strongly with acid dyes due to the affinity of the cationic groups of the proteins for the anionic reactive groups of the acid dyes. Van Gieson is a compound solution of acid dyes, developed in 1899, which can be used to selectively stain collagen red/pink and other tissues yellow. The cell nuclei are again stained blue-black by the use of haematoxylin. For HVG staining, samples were taken down to water and stain with 1% w/v celestin blue (Merck Ltd, UK) for 5min, then drained and stained with Mayer's haematoxylin (Merck Ltd, UK) for 5min, rinsed in tap water, rapidly differentiated with 1% w/v hydrochloric acid (Merck Ltd, UK) in 70% IMS 99 (Genta Medical, UK), washed in tap water for 5min, then rinsed in distilled water. Samples were then stained with Van Gieson's working solution (9 parts saturated aqueous picric acid (Merck Ltd, UK): 1 part 1% w/v aqueous acid fuchsin (Merck Ltd, UK)) for 3min, rinsed rapidly in 100% IMS 99 then immersed in xylene for up to 4h before application of cover slips.

### 5.14.2. Immunochemistry

Immunocytochemistry (ICC) refers to the staining of cell monolayers whereas immunohistochemistry (IHC) refers to the staining of tissue sections. IHC and ICC are techniques used to identify cellular and tissue constituents (antigens) by means of antigen-antibody interactions. The site of antibody binding is either identified by direct labeling or the use of a secondary labeling method. An advantage of an indirect method, such as the peroxidase-anti-peroxidase (PAP) method, is that it gives increased staining when compared with direct methods. In the PAP method a specific region of an antigen of interest, known as an epitope, is recognized by the primary antibody. A PAP complex then binds to the primary antibody which is labeled with horse raddish peroxidase. This enzyme is the most commonly used label in ICC and IHC, and when incubated with the chromogen 3,3'-diaminobenzidine (DAB) it produces a stable, non-soluble, brown coloured reaction end-product which can be visualised by light microscopy (Miller 2002). IHC and ICC are useful for ascertaining semi-quantitatively the level of gene expression and the distribution of a protein in a sample.

### 5.14.3. Sample preparations

10% v/v formal-saline was prepared by dissolving 10ml 40% w/v formaldehyde (Sigma, UK) in 90ml 0.85% w/v saline solution (Sigma, UK). This was used to fix 3D samples for 24h at room temperature. Acetone was obtained form Sigma, UK and used to fix cell monolayers for 30min at room temperature.

3D samples were transferred to CellSafe biopsy inserts (CellPath Ltd, Newtown, UK) and placed in cassettes (CellPath Ltd, UK). They were automatically processed by progressive dehydration in graded ethanol then immersed in xylene using a Citadel 1000 Tissue Processor (Thermo Scientific Inc, UK). Samples were then transferred to liquid paraffin (Tissue-Tek III Embedding Wax (polymer added), Sakura, UK) and embedded in paraffin using a Tissue-Tek TEC Tissue Embedding Console System (Sakura, UK).

Antibodies, dilutions and suppliers are displayed in Table 5.1. Haematoxyllins, eosin, and other stains for histology were obtained from Surgipath Europe Ltd, UK.

**Table 5.1: Antibodies used in Immunohistological and immunocytochemical experiments.**

| Antibody | Supplier | Dilution Used | Antigen Retrieval Method |
|---|---|---|---|
| Proliferating cell nuclear Antigen (PCNA) | Dako (Cambridgeshire, UK) | 1/10 | N/A |
| Pan Cytokeratin (PanK) | Sigma (Poole, UK) | 1/400 | 30min in trypsin at room temperature. |

Embedded samples were cooled to 5°C in a refrigerator then placed on ice before 5µm sections were cut using a microtome (Leica RM 2035, Bucks, UK) and attached to glass slides for histology or Superfrost Plus glass slides (Laboratory Sales Limited, Rochdale, UK) for immunohistochemistry (IHC) . Slides were dried at 56°C for 2h for IHC and for 45min at 60°C for histology. The wax was then removed from the sections by immersion of the slides in xylene then samples were progressively rehydrated through graded ethanol. For IHC, all samples were labelled according to the peroxidase-anti-peroxidase (PAP) method, for which no antigen retrieval was necessary, and counterstained with Mayer's Haematoxylin (Surgipath Europe Ltd, Peterborough, UK). Coverslips were then mounted on the slides using Xam (BDH Laboratory Supplies, Poole, UK) and viewed with a light microscope. The

images were then corrected using Image J software (NIH, Maryland, USA) to remove any uneven illumination from the image.

### 5.15. Semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR)

RNeasy mini kits (Qiagen, UK) and DNase kits (Qiagen, UK) were used to extract sample RNA. For lysis buffer preparation, beta-mercaptoethanol (Promega, UK), a reducing agent used for nuclease inhibition, was added to the RLT buffer (supplied in the RNeasy kit at a ratio of 1/100 (v/v). RNA isolation was performed according to the instructions in the Qiagen RNeasy mini kit. mRNA quality and quantity were evaluated and quantified using a spectrophotometer (BioPhotometer, Eppendorf, UK) and by electrophoresis using a 1% w/v agarose gel, prepared by dissolving agarose in Tris Acetate EDTA (TAE) buffer. 3µl of stock solution of 10mg/ml ethidium bromide (Promega, UK) were added to 120ml gel solution to allow visualisation of bands on the gel when exposed to fluorescent light. The gel was poured into a mold and combs placed in the gel to create the wells and left for around 20min at room temperature until the gel solidified. The gel was immersed in TAE buffer in the electrophoresis tank. HyperLadder IV (Bioline, UK) was loaded onto the gel to provide molecular weight markers. Cell lysates and mRNA samples were stored at -20°C and -80°C respectively to prevent sample degradation, and both were kept on crushed ice throughout the experiments.

Omniscript reverse transcriptase, oligo dT primer (0.5ug/ml) and RNase inhibitor (40units/ul) from Promega (UK) were used for reverse transcription of mRNA to complementary DNA (cDNA) according to the instructions in the Omniscript kit handbook. Montage PCR centrifugal filters (Millipore, UK) were applied to purify the cDNA. The cDNA and mRNA quality and quantity were evaluated using a spectrophotometer (BioPhotometer, Eppendorf, UK) and by running samples on a 1.5% w/v agarose electrophoresis gel with ethidium bromide. Mastercycler thermal cycler (Eppendorf, UK) and REDTaq® ReadyMix$^{TM}$ PCR reaction mixture was used to amplify the cDNA according to the manufacturer's instructions. The PCR program was as follows; 94 °C for 5 min, denature the template at 94°C for 20sec, anneal primers at 60°C for 20sec, Extension at 72°C for 20sec, 72°C for 10min. 30 cycles were performed then samples were then held at 4ºC. All primers were designed using Primer 3 and obtained from Invitrogen, UK. The primers used are summarised in Table 5.2. The expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was normalised between samples in each set, so that the relative expression of genes of interest could be established.

GAPDH was used for normalisation since it a house keeping gene and the expression of GAPDH should be relatively constant and, therefore, be proportional to cell number.

The cDNA samples were stored at -20°C at kept on crushed ice throughout the experiments to inhibit nuclease activity. All solutions and samples were prepared using RNase-free water and nuclease free plastic ware (Appleton Woods, UK). The plastic ware was autoclaved before use and care was taken not to introduce contaminants to the plastic ware throughout the experiments. Electrophoresis tanks and combs were cleaned with detergent and rinsed thoroughly in double distilled water between uses.

**Table 5.2: Primers used for semi-quantitative RT-PCR experiments.**

| Primer | Gene | Primers (Forward(F) and Reverse (R)) | Product length (bps) |
|---|---|---|---|
| rGAPDH | Rat GAPDH | F-CCCATCACCATCTTCCAGGAG<br>R-GCTGAACGGGAAGCTCACTGG | 227 |
| rINV | Rat involucrin | F-AAGTCCCCAGAGCCAGAACT<br>R-TGTGAGTCATCCAGCTCCTG | 308 |
| rK5 | Rat cytokeratin 5 | F-AGTGTGCCAACCTCCAGAAT<br>R-AGCCATAGCCAATGTTGCTT | 318 |
| rK10 | Rat cytokeratin 10 | F-TCACCACAGAAATCGACAGC<br>R-GGGTTATTTGCAGGTTTCCA | 360 |
| rK1 | Rat cytokeratin 1 | F-CCCACCACACCATTAGCTCT<br>R-GCGCTCCGTAAAAGACACTC | 369 |
| mKGF | Mouse keratinocyte growth factor | F-GATCAAGCCCCACACAAACT<br>R-TGCTAGTCATGGTGTCAGCC | 611 |
| mIL-6 | Mouse interleukin 6 | F-CCGGAGAGGAGACTTCACAG<br>R-GGAAATTGGGGTAGGAAGGA | 339 |
| mVEGF | Mouse vascular endothelial growth factor | F-CTGCTCTGGGTCCACTGG<br>R-CACCGGGTTGGCTTGTCACAT | 431, 563, 635 |
| mFGF-2 | Mouse fibroblast growth factor - 2 | F-AGCGGCTCTACTGCAAGAAC<br>R-TATGGCCTTCTGTCCAGGTC | 350 |
| mNGF | Mouse nerve growth factor | F-TGTGCCTCAAGCCAGTGAAATT<br>R-TCCACAGTGATGTTGCGGGTCT | 463 |
| mGAPDH | Mouse GAPDH | F-CCCATCACCATCTTCCAGGAGC<br>R-CCAGTGAGCTTCCCGTTCAGC | 450 |

### 5.16. Statistical Analysis

Statistical significance ($P<0.05$) between test groups was determined by one-way analysis of variance (ANOVA) and Tukey post-hoc test (SPSS v.17, Chicago, USA).

# 6. Effect of encapsulation on fibroblast viability, growth and catabolic activity

Alginate hydrogel was used to encapsulate 3T3 fibroblasts to tissue engineer the dermis, in which the 3T3 murine fibroblasts serve as analogues for autologous dermal fibroblasts. This alginate/ fibroblast graft will be implanted into the wound site to facilitate replacement of the lost dermis, and to act as a 'feeder layer' to support the growth and differentiation of keratinocytes on the scaffold surface to form a new epidermis. The effect that the alginate hydrogel scaffold has on the behaviour of cells is important for the success of the graft since scaffolds play an important part in controlling cell behaviour including growth (Brandl *et al.* 2007; Discher *et al*. 2005; Kakisis *et al*. 2005). It is desirable to limit proliferation of the encapsulated fibroblasts to prevent over-growth of keratinocytes and so that the viability of fibroblasts is maintained within the initially avascular scaffold.

When a tissue engineered (TE) construct is delivered to the body, meeting the oxygen requirement of the cells is essential for the success of the treatment (Nomi *et al*. 2002), but also later to support various cellular mechanisms, including cell cycle, cell proliferation, glucose metabolism and cell differentiation (Mishra & Starly 2009). This is more likely to be achieved if the early cell density, and therefore oxygen requirement of the avascular graft remains relatively low (Mishra & Starly 2009).

For production of the TE skin a co-culture of keratinocytes and fibroblasts is used. Fibroblasts have a higher mitotic index than keratinocytes so to prevent overgrowth these cells are usually mitotically inhibited. The growth of the fibroblasts can be inhibited by gamma irradiation or Mitomycin C treatment (Stanovic *et al*. 2000; Tseng *et al*. 1996). These approaches are, however, only suitable for production of TE epidermal layers *in vitro* since they are lethal to the cells. The mitotically inhibited fibroblasts can be intermittently replaced to allow them to act as a feeder layer to support keratinocytes, *in vitro*. Since the TE skin under development is, however, intended to be implanted and allow *in vivo* tissue development of both the epidermis and the dermis, it is essential that the fibroblasts remain both mitotically inhibited and viable to act both initially as a feeder layer for keratinocytes, and also, later to facilitate dermal repair.

Cell proliferation is known to be controlled by cell-cell contact (Wieser *et al*. 1990; Wieser & Oesch 1995). Isolating cells to reduce cell-cell contact in both 2D and 3D culture has been shown to limit cell proliferation (Liu *et al*. 2006b; Mishra & Starly 2009). Reducing cell density has also been shown to reduce catabolic activity and therefore the oxygen requirement of each cell (Guarino *et al*. 2004). Limiting cell-cell contact in the 3D environment of the alginate hydrogels should achieve the same reduced proliferation and catabolic activity that is seen in monolayer culture, whilst maintaining cell viability. In order to reduce cell-cell contact in the alginate hydrogels it is important to mechanically isolate the fibroblasts from one other as adherent cells tend to adhere to each other and form dense aggregates. Such aggregates may develop central necrotic regions due to the relative inaccessibility of nutrients and oxygen to cells embedded in the core, or due to the build up of toxic products within the core (Guarino *et al.* 2004). In this chapter the viability, growth, catabolic activity and change in cell distribution exhibited by fibroblasts encapsulated as uniformly dispersed entities in two concentrations of low viscosity calcium-alginate are described.

## 6.1. Results

### 6.1.1. Live/ dead staining

Calcein-AM/ PI Live/ dead staining was used to assess the long-term effect of alginate hydrogel encapsulation on cell viability. *In situ* live/dead staining of fibroblasts encapsulated in 2% and 5% w/v alginate was performed at 3, 14, 28, 60 and 150 days. The cells were seen to be homogeneously distributed throughout the alginate hydrogels as dispersed entities rather than aggregates at all time points in both 2% and 5% w/v alginate hydrogels. Sections from the centre of alginate beads or discs of 3mm diameter are shown in Figure 6.1. The presence of mainly green and very few red cells illustrated that cell viability was maintained throughout the hydrogels for at least 150 days *in vitro* culture.

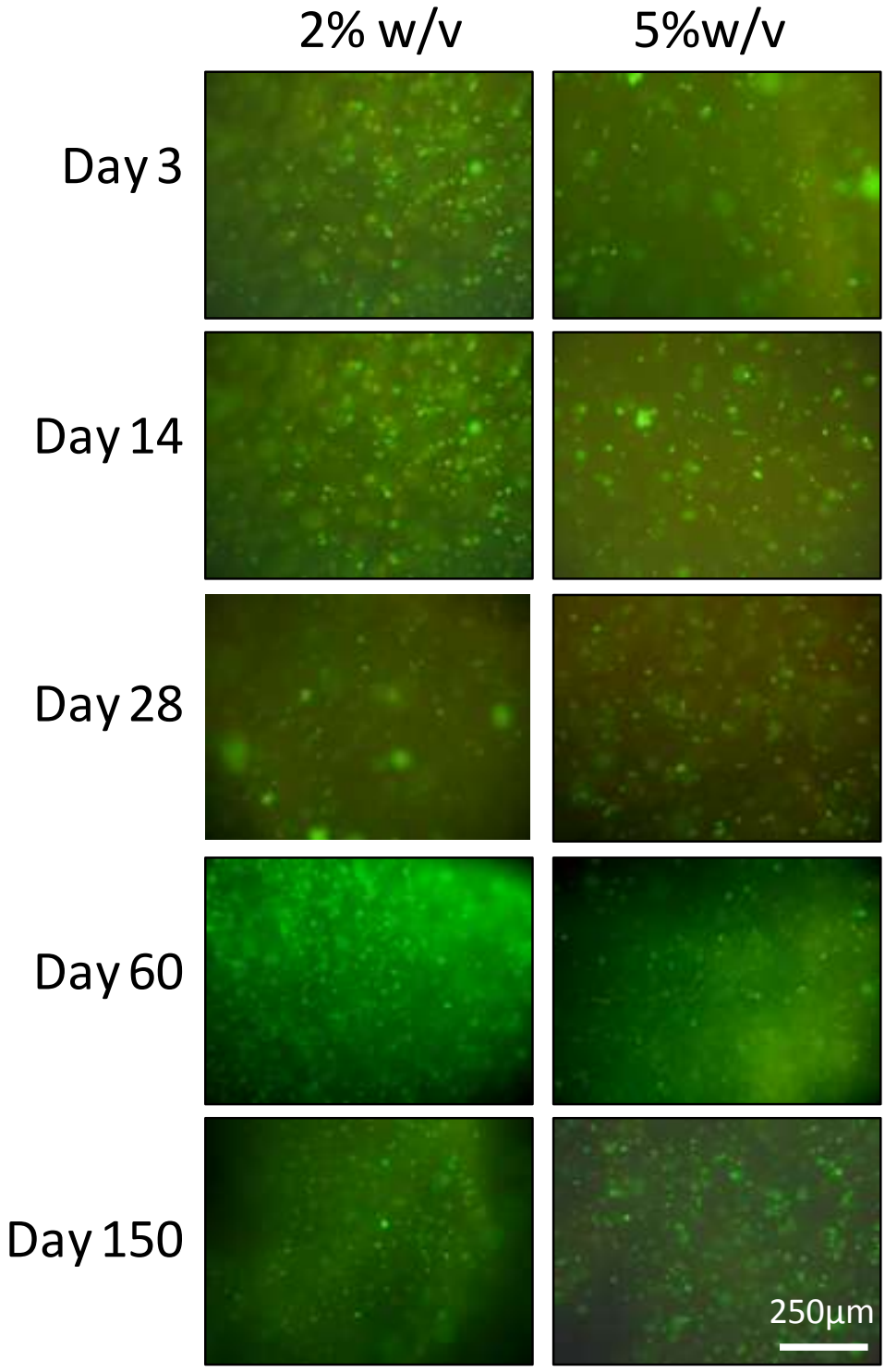



**Figure 6.1: Alginate encapsulated fibroblasts stained with Calcein AM/ PI live/ dead stain in 2% w/v and 5% w/v alginate at 3, 14, 28, 60 and 150 days post encapsulation. No evidence of spheroid formation or cell proliferation is visible. The viability of cells throughout the experiment was illustrated by the presence of mainly green cells and very few red cells. This indicates that the diffusion of nutrients and waste products through the matrix was sufficient to maintain the cells' viability.**

### 6.1.2. Effect of culture time on fibroblast distribution within the alginate hydrogels

Histological evaluation of the encapsulated cells in 2% w/v and 5% w/v alginate using H&E (Figure 6.2) illustrated the architecture of the construct by staining the cytoplasm of cells pink due to eosin and the cell nuclei and alginate chains blue/black by the haematoxylin due to the negative charges of the nuclei and the alginate chains. Sections of the tissue engineered construct were taken weekly from week one to week five. This staining illustrated that the encapsulated cells remained present within the scaffold as discrete entities and not as aggregates throughout the duration of the study, mechanically confined from each other by the alginate hydrogel surrounding the individual cells in both 2% and 5% w/v alginate hydrogels (Figure 6.2).

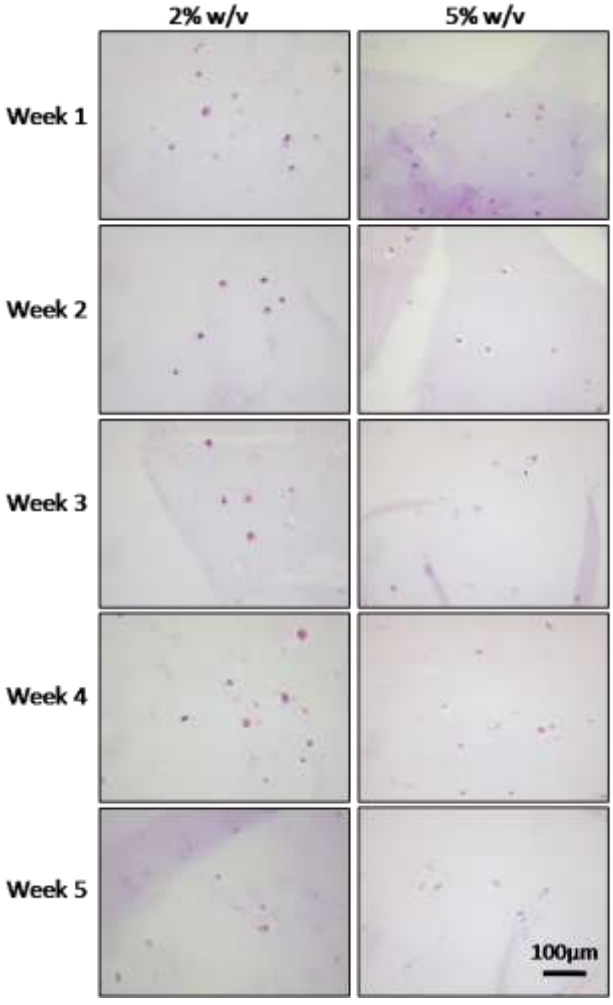
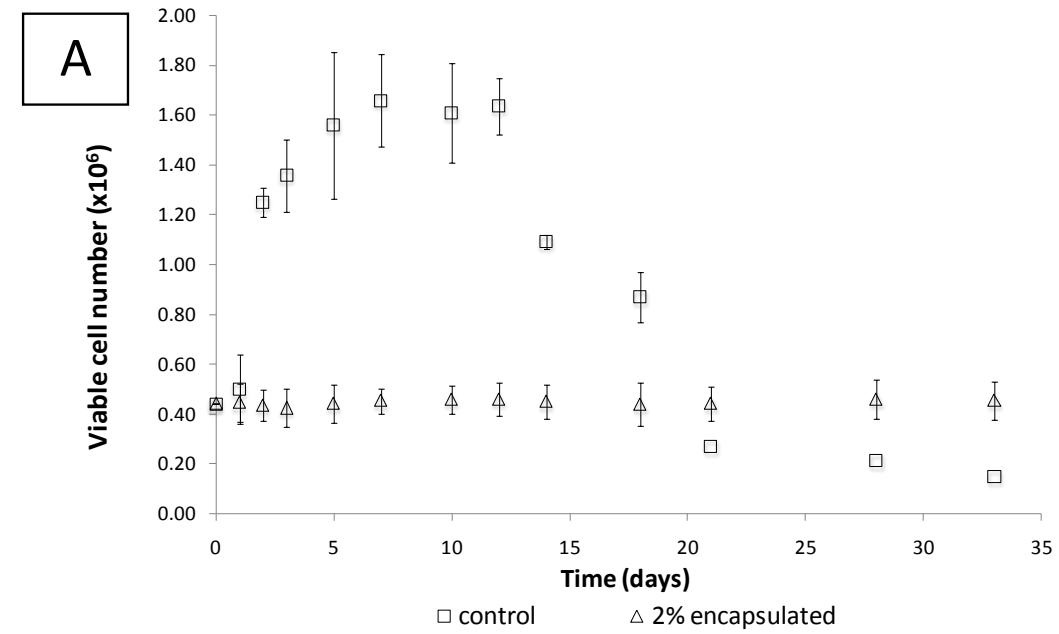



**Figure 6.2: H&E staining of encapsulated fibroblasts up to five weeks post-encapsulation in 2% w/v alginate and 5% w/v alginate. Cell nuclei and alginate stain blue/ black and cytoplasm of cells stain pink. The cells are shown to remain as dispersed entities mechanically confined by the alginate hydrogel throughout the experiment.**

### 6.1.3. Effect of alginate encapsulation on fibroblast proliferation

The effect that encapsulation in 2% and 5% w/v alginate hydrogels had on mitotic activity of cells both during their encapsulation and following their release from the degraded alginate hydrogels was investigated using the MTT assay. This assay relies on mitochondrial activity of viable cells to covert MTT to formazan and can be used to quantitatively measure viable cell numbers encapsulated in alginate (Khattak *et al*. 2006).

Fibroblasts encapsulated in 2% and 5% w/v alginate hydrogels were shown to remain viable but be mitotically inhibited by the encapsulation, as illustrated by the lack of change in viable cell number throughout the 33 days from the cell seeding number of $4.4\times10^5$ cells (Figure 6.3). On release from 2% and 5% w/v alginate, the cells were shown to attach to tissue culture plastic and proliferate in monolayer culture after one and three weeks encapsulation in a similar manner to that seen before encapsulation (Figure 6.4). The bell-shaped growth curve that is seen in these monolayer cultures is due to proliferation of the cells in the first few days to cover the surface of the culture dish to confluence, when the rate of cell proliferation decreases. Cell number is then maintained as the cells enter the stationary phase (Freshney 2005). Subsequently, cell death begins to occur at a higher rate than proliferation, as nutrients likely become limited (Hwang & Lee 2008) and toxic waste products likely begin to accumulate, since the medium was only refreshed three times weekly. Dying cells release pro-apoptotic growth factors and cytokines (Burscha *et al*. 2008), and this would induce the death of surrounding cells.



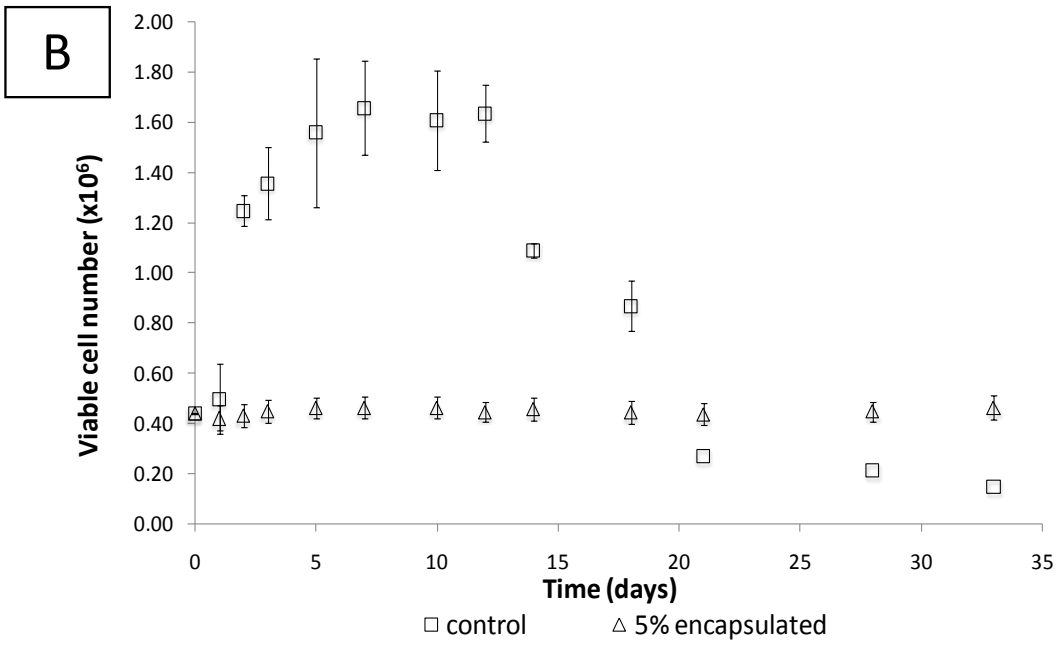




**Figure 6.3: Growth curves determined by the MTT growh assay of 3T3 fibroblasts encapsulated in a) 2% and b) 5% w/v alginate hydrogels, compared with non-encapsulated 3T3s (control). Mean values ± SD are shown (n=3). The fibroblasts encapsulated in 2% and 5% w/v alginate remain at a constant cell number up to 33 days post-encapsulation, rather than exhibiting the bell-shaped growth curve seen with monolayer cultured fibroblasts. Significant differences ($p<0.05$) between encapsualed samples and the control were seen at all time points except day 0 and 1. No significant differences were seen between samples encapsulated in 2% and 5% w/v alginate hydrogels.**

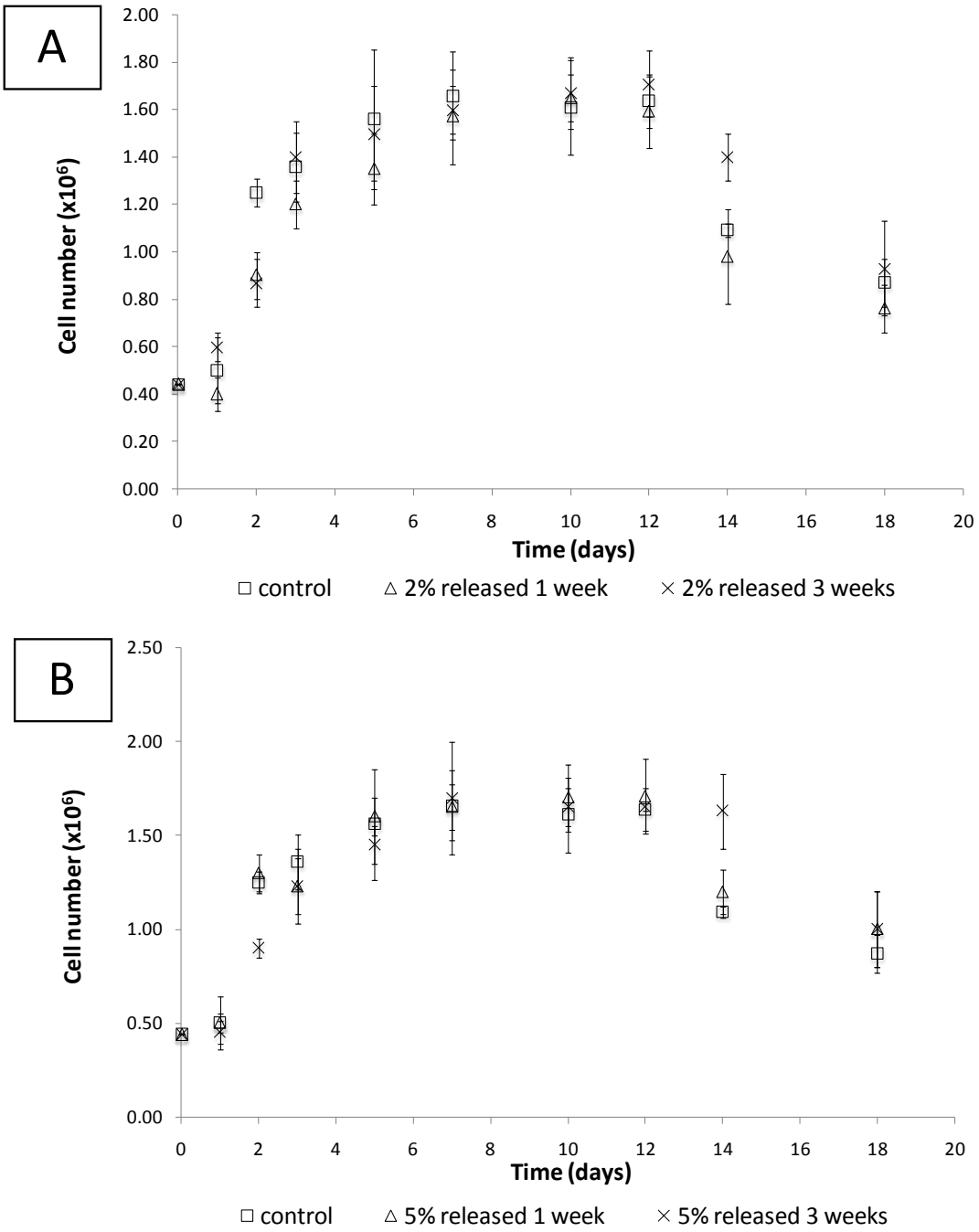



**Figure 6.4: Growth curves determined by the MTT growh assay of 3T3 fibroblasts released after one and three weeks encapsulation in a) 2% and b) 5% w/v alginate compared with non-encapsulated 3T3s (control). Mean values ± SD are shown (n=3). Fibroblasts released from encapsulation in 2% and 5% w/v alginate generally displayed normal growth in monolayer culture after both one and three weeks encapsulation. Significant differences ($p<0.05$) between controls and released samples were only seen at days three (2% one and three week samples; 5% three week sample) and day 14 (2% and 5% at three week samples).**

The possible causes of the reversible mitotic inhibition demonstrated by cells encapsulated in alginate hydrogel were investigated by examining the growth of fibroblasts in different conditions. 3T3 fibroblasts grown in monolayer culture in alginate hydrogel presence were shown to exhibit normal mitotic activity when compared with non-encapsulated control samples. 3T3 fibroblasts grown in monolayer culture after exposure to 100mM calcium chloride and 100mM tri-sodium citrate for a period of 2h also displayed normal mitotic behaviour (Figure 6.5).

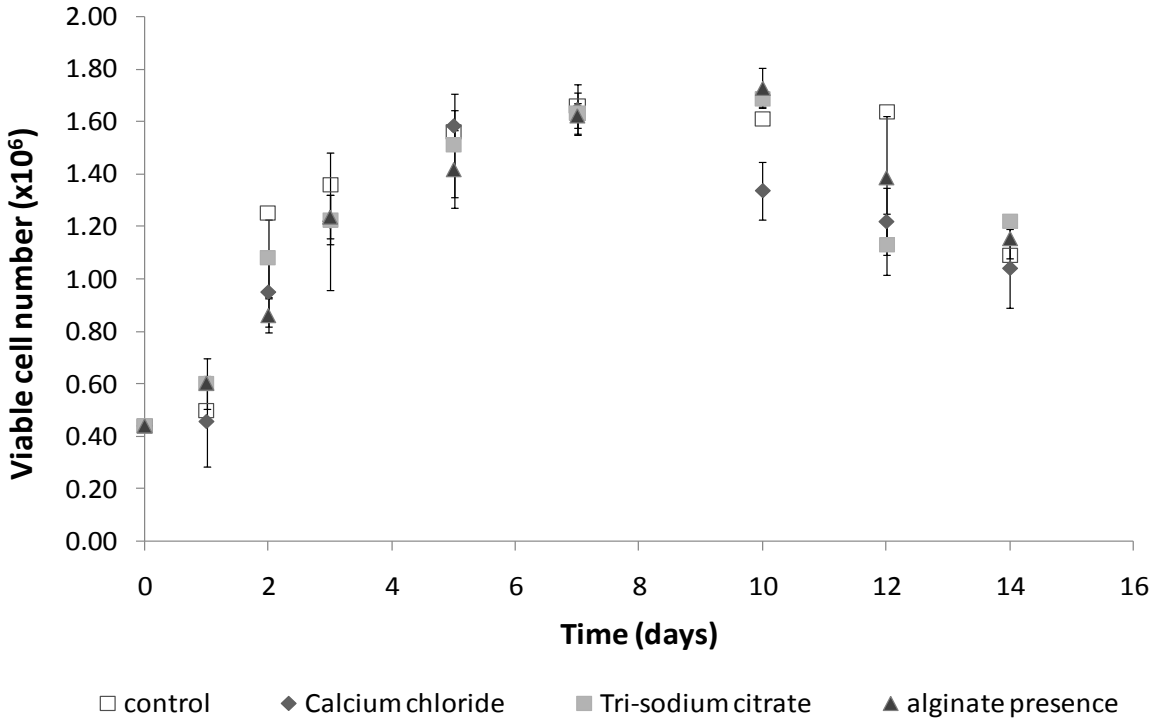



**Figure 6.5: Growth curves determined by the MTT growh assay of non-encapsulated fibroblasts in the presence of 5% w/v alginate hydrogel, treated with $CaCl_2$ and treated with tri-sodium citrate, compared with non-encapsulated control samples. Mean values ± SD are shown (n=3). The growth curves were seen to be very similar under all conditions showing that alginate presence, citrate treatment and $CaCl_2$ treatment had no appreciable effect on cell proliferation. Compared with the control, significant differences ($p<0.5$) were only seen at 3h for $CaCl_2$ and alginate presence and for all samples at 12h.**

The reversible mitotic inhibition exhibited by cells encapsulated in alginate hydrogels was confirmed by staining for PCNA. PCNA is a marker expressed primarily in the nuclei of cells in S phase of the cell cycle and thus can be used as a measure of mitotic activity (Foley

*et al*. 1991; Leong *et al*. 1995). Before encapsulation in alginate, 3T3 fibroblasts were shown to express PCNA when grown in monolayer culture, as shown by the intense brown staining of cell nuclei (Figure 6.6a). Fibroblasts are the main cell type present in the dermis of skin (MacNeil 2007) and the majority of cells encapsulated in the dermis of skin stained blue with the haematoxylin counter-stain, rather than brown and, therefore, were not dividing (Figure 6.6b). PCNA staining of encapsulated fibroblasts in 2% and 5% w/v alginate confirmed that cells were not mitotically active at either week one or week three post-encapsulation (Figure 6.7), like fibroblasts encapsulated in the ECM of normal rat dermis (Figure 6.6b). PCNA staining also confirmed that when fibroblasts were released from the alginate hydrogels, normal mitotic activity was resumed, with nearly 100% of the cells staining positively for PCNA (Figure 6.7), as seen before encapsulation (Figure 6.6a).

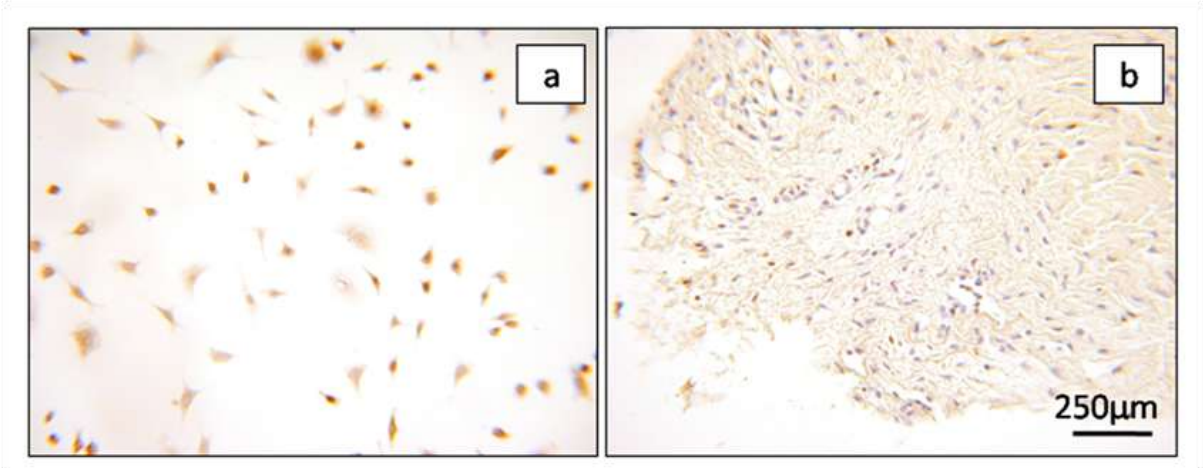



**Figure 6.6: PCNA expression in a) non-encapsulated (control) 3T3 fibroblasts and b) fibroblasts in the rat dermis. Cells which express PCNA and are dividing have strongly stained brown nuclei, whereas cells which were not dividing and did not express PCNA stained blue due to the haematoxylin counter-stain. The majority of fibroblasts in the dermis did not express PCNA whereas the fibroblasts in monolayer culture express PCNA.**

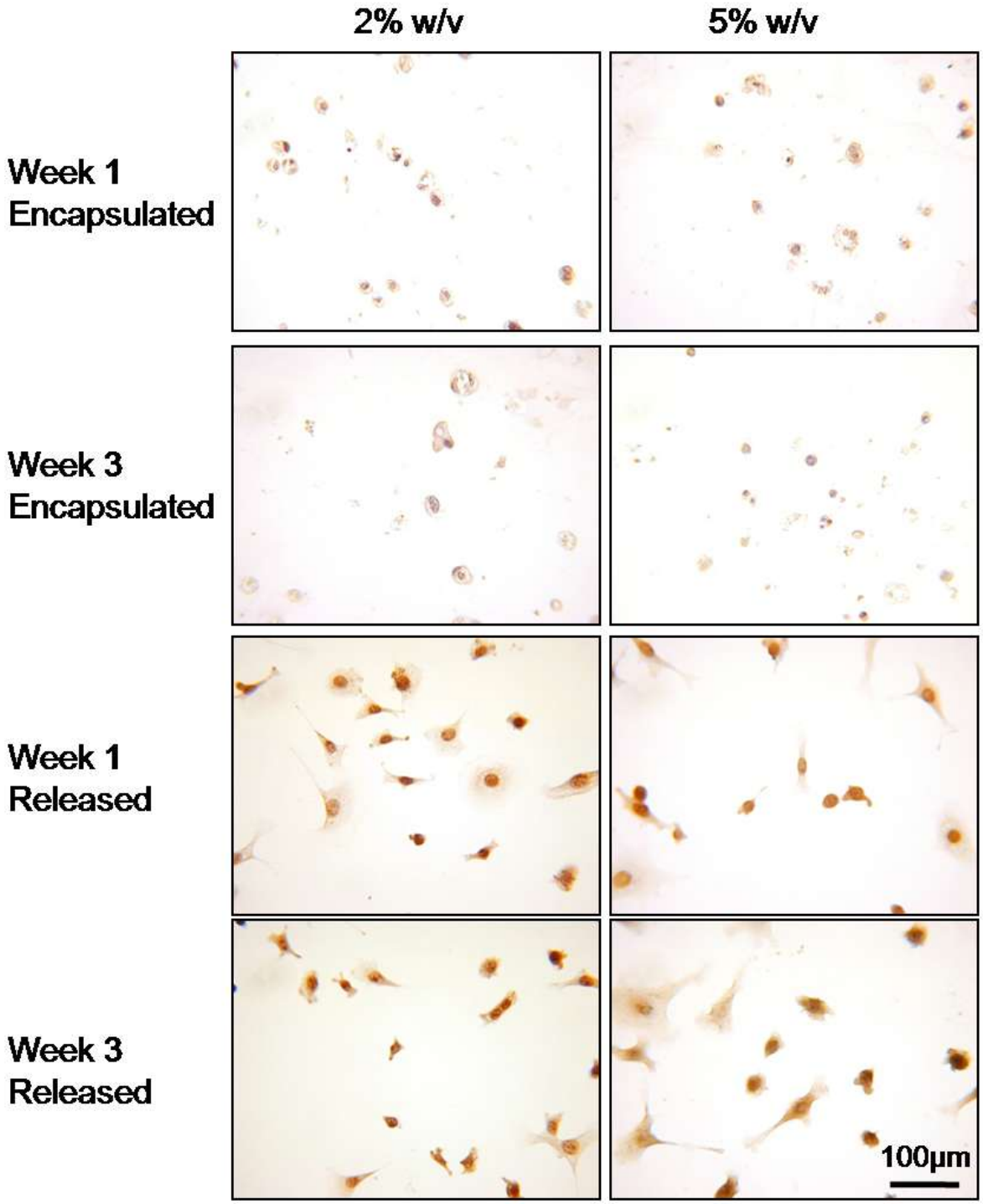



**Figure 6.7: PCNA expression of encapsulated 3T3 cells encapsulated and released from 2% w/v and 5% w/v alginate after one and three weeks encapsulation. All encapsulated fibroblasts were shown to have blue nuclei and therefore did not express PCNA and were not dividing and all released fibroblasts were shown to express PCNA and be proliferating.**

### 6.1.4. Effect of alginate encapsulation on catabolic activity of cells

The catabolic activity of fibroblasts were determined by measuring the amount of formazan that was produced (from reduction of MTT) with time. Before encapsulation, $3.75\times10^5$ fibroblasts in monolayer culture were shown to produce 5.37±0.37µg of formazan from reduction of MTT in 5h, when no further formazan was produced (Figure 6.8a). In comparison, the same number of cells encapsulated in 2% w/v alginate were shown to only produce 0.65±0.03µg of formazan in 5h after one week encapsulation and formazan continued to be produced between 5h and 11h (Figure 6.8a). After three weeks encapsulation in 2% w/v alginate, the amount of formazan produced with time was not significantly different from that seen after one week encapsulation ($p<0.05$) (Figure 6.8a). Fibroblasts encapsulated in 5% w/v alginate also exhibited a reduced ability to reduce MTT to formazan after both one and three weeks encapsulation when compared with non-encapsulated control fibroblasts (Figure 6.9b). When fibroblasts were released form 2% w/v alginate (Figure 6.9a) and 5% w/v alginate (Figure 6.9b) after one and three weeks encapsulation, the cells were shown to reduce MTT to formazan at a similar rate to that seen before encapsulation showing that the catabolic inhibition seen during encapsulation was reversible.

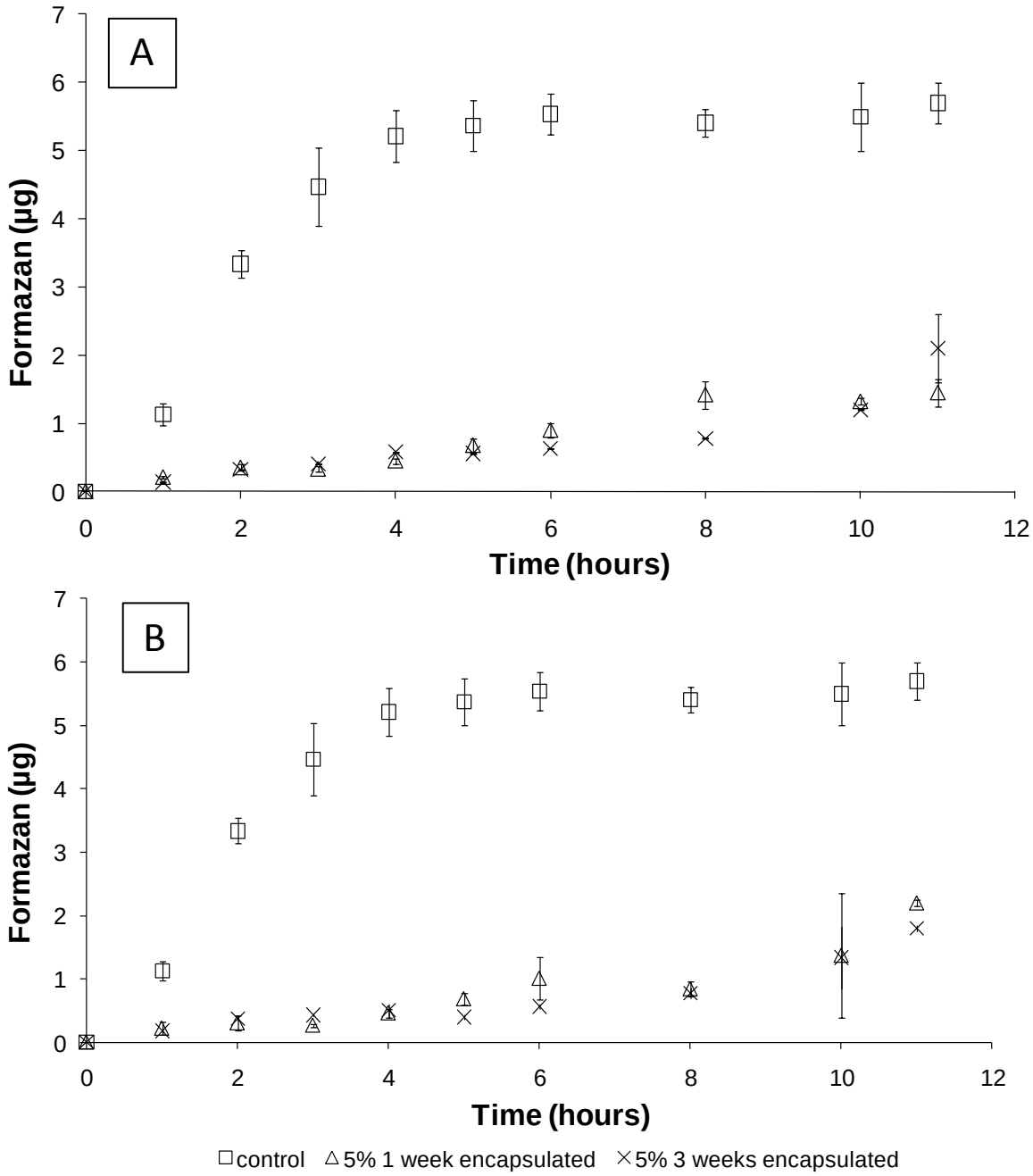



**Figure 6.8: Formazan produced with time from MTT for non-encapsulated (control) fibroblasts compared to fibroblasts encapsulated in a) 2% w/v and b) 5% w/v alginate hydrogel at one and three weeks post-encapsulation. Mean values ± SD are shown (n=3). The rate at which the fibroblasts produce formazan was comparable for encapsulated cells at both one and three weeks post encapsulation. The amount of formazan produced by both 5% w/v encapsulated fibroblast samples was significantly lower ($p<0.05$) than by control samples at all time points.**

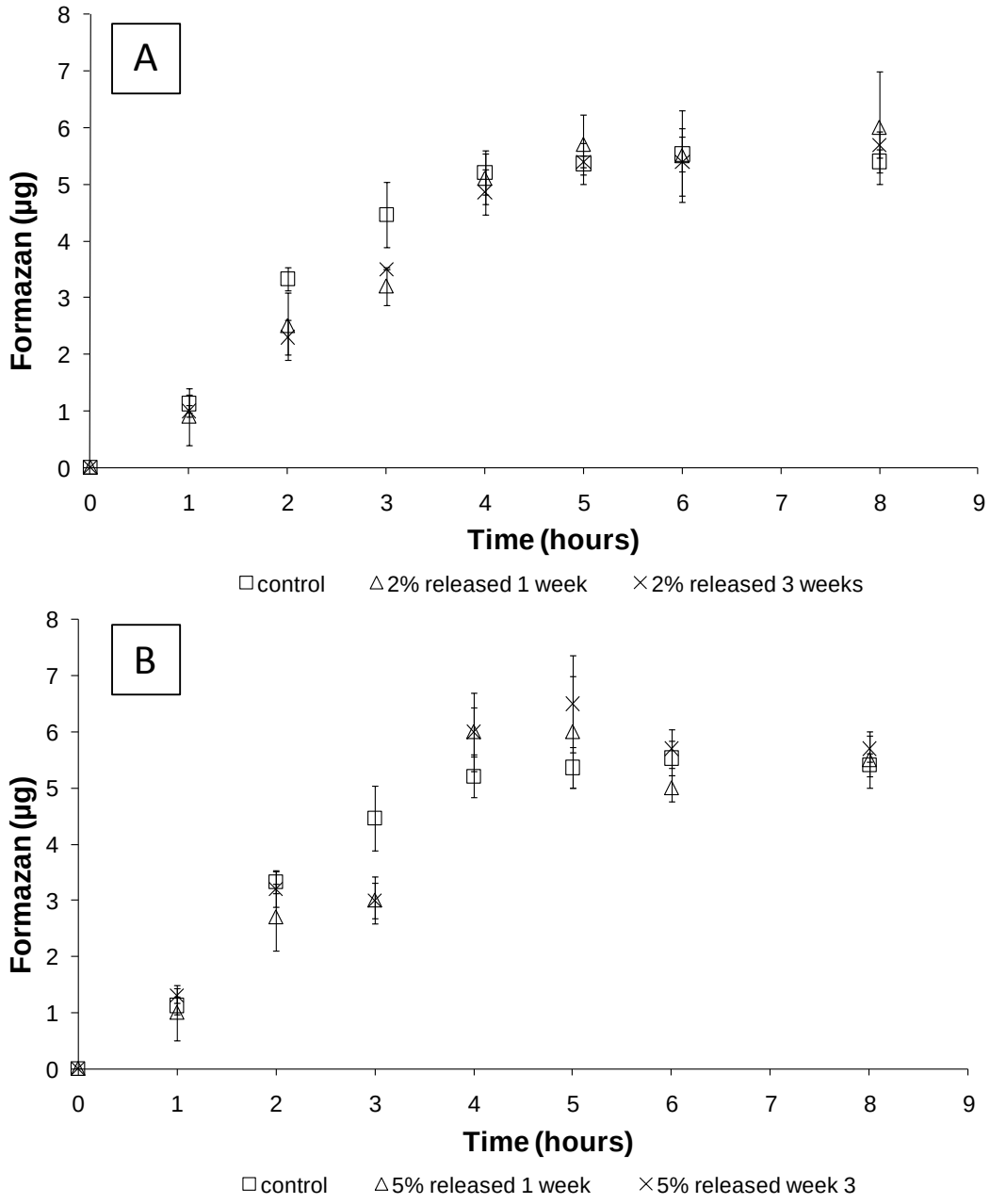



**Figure 6.9: Formazan produced with time from MTT for non-encapsulated (control) fibroblasts compared to fibroblasts released from a) 2% w/v and b) 5% w/v alginate hydrogels at one and three weeks post-encapsulation. Mean values ± SD are shown (n=3). The rate at which the fibroblasts produce formazan was comparable for all samples showing that after release from encapsulation in 2% w/v and 5% w/v alginate the fibroblasts resumed normal catabolic activity. Significant differences (compared with the control) ($p<0.05$) were only seen at 3h for 2% three week samples and 5% one and three week samples, and at 5h for 5% three week samples.**

Calcium chloride treatment, tri-sodium citrate treatment and the presence of alginate hydrogel were shown to have no appreciable effect on the ability of fibroblasts in monolayer culture to reduce MTT to formazan (Figure 6.10). This showed that these factors were not responsible for the reversible catabolic inhibition which is seen during encapsulation in 2% and 5% w/v alginate hydrogels.

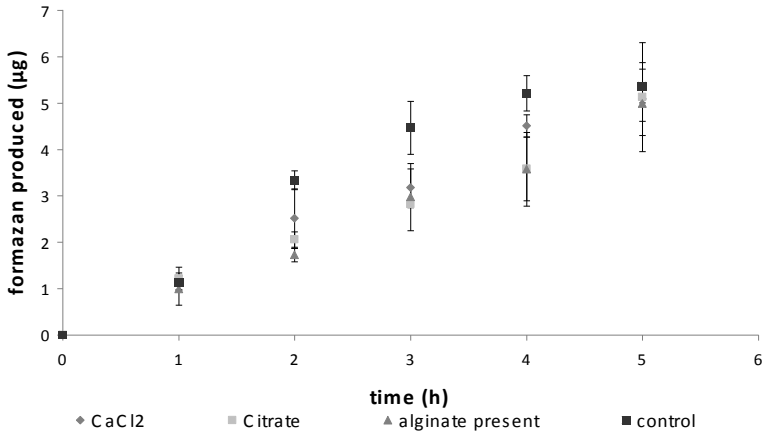



**Figure 6.10: Formazan produced with time from MTT for non-encapsulated (control) fibroblasts compared with non-encapsulated cells exposed to calcium chloride or tri-sodium citrate or cultured in the presence of 5% w/v alginate hydrogel. Mean values ± SD are shown (n=3). The rate of formazan production was comparible between all four samples, although the amount of formazan produced by all three samples at 2h and 3h, and alginate presence and citrate samples at 4h was significantly lower ($p<0.05$) than the control.**

The reversible catabolic inhibition by encapsulation was confirmed by staining the cells with JC-1, which gives a measure of mitochondrial membrane potential. Fibroblasts encapsulated in both 2% and 5% w/v alginate after one and three weeks encapsulation were shown to exhibit reduced red fluorescence (Figure 6.11) when compared with control cells (Figure 6.12a) indicating that encapsulated cells had a lower mitochondrial activity than control samples. The amount of red fluorescence was observed to be comparable for all encapsulated samples showing that incubation time and percentage alginate had no effect on the cells' catabolic activity. Cells released from 2% and 5% w/v alginate after one and three weeks encapsulation (Figure 6.12b-e) were observed to exhibit a level of red fluorescence which was comparable with that which was seen before encapsulation (Figure 6.12a). This showed that after release from encapsulation the fibroblasts resumed normal catabolic activity.

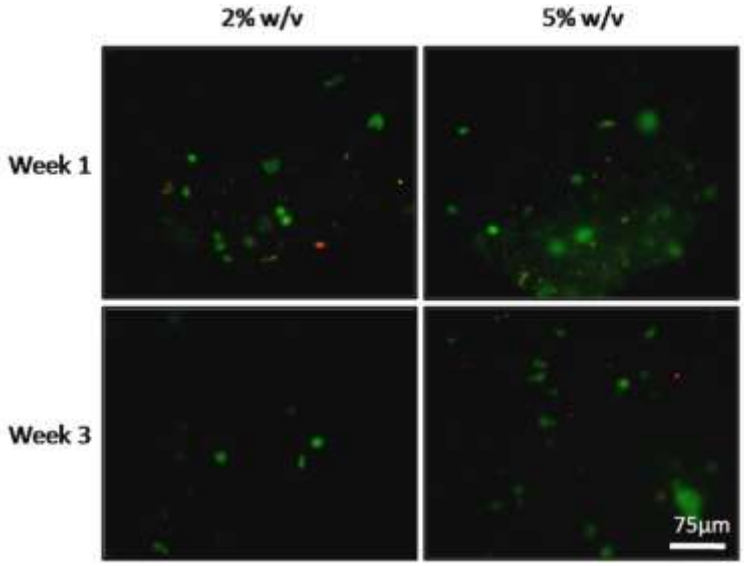



**Figure 6.11: JC-1 staining of encapsulated fibroblasts in 2% and 5% w/v alginate one and three weeks post encapsulation. Mitochondria with a high membrane potential stain red in catabolically active cells stain red. There were very few cells with red staining present in any of the photomicrographs indicating that the cells were not very catabolically active when encapsulated in alginate hydrogels.**

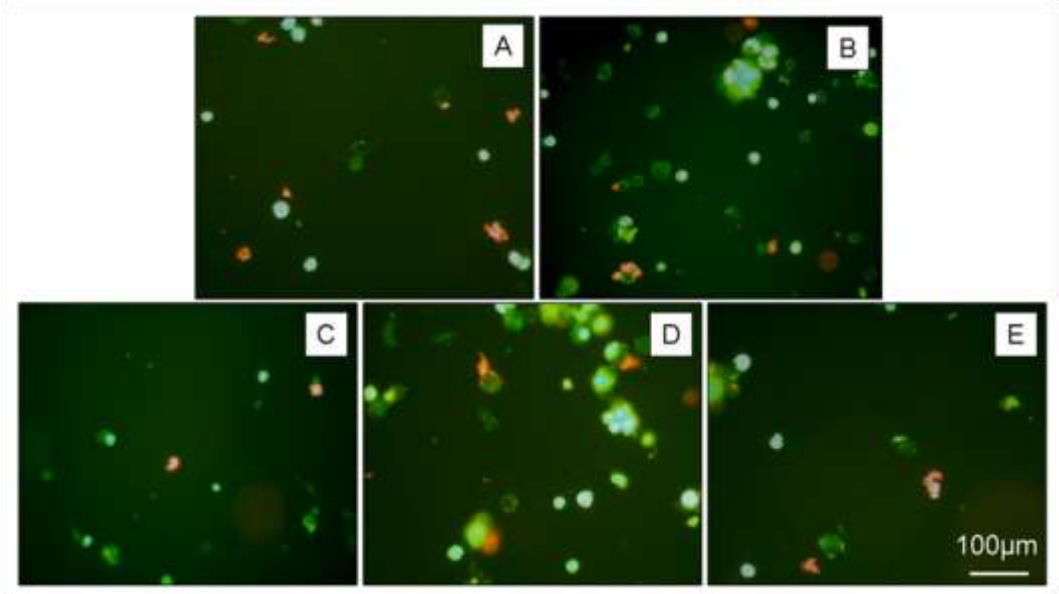



**Figure 6.12: JC-1 staining of fibroblast monolayers a) before encapsulation (control), b) released form 2% alginate after one week, c) released from 2% alginate after three weeks, d) released from 5% alginate after one week and e) released from 5% alginate after three weeks. Mitochondria with a high membrane potential in catabolically active cells stain red. In all samples the number of cells with some or complete red staining (catabolically active) was similar showing that after the cells are released from alginates they resume normal catabolic activity.**

## 6.2. Discussion

The encapsulated fibroblasts were seen to be homogeneous distribution throughout the alginate hydrogels at all time points. This contradicted the hypothesis made by Bienaime *et al.* (2003) that cell distribution would be more concentrated towards the periphery of the alginate hydrogels. The authors proposed that this would happen due to the movement of alginate chains to the periphery during the gelling process, which would pull the cells toward the periphery. Furthermore, the authors hypothesised that the effect would be especially evident when the concentration of alginate hydrocolloid was increased, and this was also not evident upon increasing the concentration from 2% to 5% w/v.

Fibroblasts encapsulated in both 2% and 5% w/v alginate hydrogels were shown to be viable up to 150 days encapsulation (Figure 6.1). The long term viability of other encapsulated cells has been demonstrated, for example with chondrocytes (Schagemann *et al*. 2006) and human embryonic stem cells (Siti-Ismail *et al*. 2008) and two other fibroblast cell lines (Chang *et al*. 1994). In the present study, the viability of cells in the centre of both 2% and 5% w/v alginate hydrogel structures with a thickness of 3mm was observed. This illustrated that there was no critical necrosis and that oxygen tension was high enough to support cell survival throughout the duration of the study, in contrast with what has been found with other alginate hydrogels and cell types (Khattak *et al*. 2007). The cells remained as viable dispersed units and failed to form cell aggregates (Figures 6.1 and 6.2) in the alginate hydrogels throughout the duration of the study, in contrast with previous reports (Khattak *et al.* 2006; Schagemann *et al.* 2006; Siti-Ismail *et al.* 2008).

Encapsulated cells in both 2% and 5% w/v alginate hydrogels were indicated to remain viable but not proliferate according to the MTT assay (Figure 6.3). The lack of proliferation was further confirmed as the encapsulated cells failed to express PCNA in the nuclei, as shown by the blue rather than brown staining of all nuclei (Figure 6.7). Following release of cells from the alginate hydrogels, normal mitotic activity was demonstrated (Figure 6.4), as was confirmed by positive staining for PCNA (Figure 6.7). This indicated that cells encapsulated in both 2% and 5% w/v alginate were reversibly mitotically inhibited. As the scaffold degrades *in vivo* by loss of calcium cross-links (Ikada 2006), the encapsulated cells will be released and should then proliferate to help to replace the lost dermal tissue at the wound site. Alginate constructs of different concentrations exhibit contrasting mechanical properties (Augst *et al.* 2006) and cell behaviour is known to be regulated by the mechanical properties

of the material on which they are cultured (Brandl *et al.* 2007;Discher *et al.* 2005). Decreasing the alginate hydrogel concentration from 5% to 2% w/v however, had no effect on the mitotic activity of encapsulated cells. This indicated that either gel would provide the reversible mitotic inhibition of encapsulated fibroblasts, which is desirable for the tissue engineering of skin *in vivo*.

It has been suggested that alginate hydrogel may affect cell proliferation either due to the release of calcium ions or the presence of alginate monomers in the media (Doyle *et al*. 1996; Lansdown 2002), however no differences in cell growth were observed for non-encapsulated cells which were cultured in the presence or absence of alginate hydrogel (Figure 6.5). This illustrated that the presence of calcium-alginate was not responsible for the lack of proliferation seen with encapsulated cells. It also indicated that non-encapsulated cells could be co-transplanted with the encapsulated cells, so that the non-encapsulated cells can proliferate *in vivo* prior to release of the encapsulated cells. It is therefore likely that the lack of mitotic activity that was exhibited by cells encapsulated in both 2% and 5% w/v alginate was due to the encapsulated cells remaining mechanically isolated from each other by the calcium cross-linked alginate hydrogel scaffold throughout the duration of the experiment (Figure 6.2). This mechanical isolation prevented cell-cell contact, which is known to modulate growth in monolayer culture (Liu *et al.* 2006). This is possibly also why the majority of fibroblasts encapsulated in the normal ECM of the dermis of skin, also failed to express PCNA (Figure 6.6b) and thus were in a quiescent state.

This study illustrates that calcium-alginate hydrogel of 2% and 5% w/v concentration effectively simulates the ECM of soft tissue allowing for nutrients, waste products and signalling molecules to effectively perfuse the scaffold to maintain viability of encapsulated fibroblasts, as has been seen with other cell types (Tan & Takeuchi 2007; Bazou *et al.* 2008). The 3D environment is known to modulate cell behaviour (Pedersen & Swartz 2005) and here we have shown that 2% and 5% w/v alginate effectively modulate cell proliferation in a similar way to normal ECM. The cell growth that has been seen in other studies (Bazou *et al.* 2008; Khattak *et al.* 2006; McConell *et al*. 2004) is possibly due to a higher cell density being used which results in greater cell-cell contact. The approach used allows for cells to remain as discrete entities within the scaffold so that no cell-cell agglomerations form. This resulted in the viable cell number remaining constant throughout the initial culture period. This should allow for sufficient proliferation of non-encapsulated co-cultured cells before the

release and subsequent proliferation of encapsulated cells. The cell isolation (Figure 6.2) was also likely to be the reason for the reduced metabolic activity seen with encapsulated fibroblasts compared with non-encapsulated control fibroblasts (Figure 6.8 and 6.11). This was assumed since alginate presence, citrate treatment and $CaCl_2$ treatment had no effect on the ability of fibroblasts to reduce MTT to formazan (Figure 6.10) and after release from encapsulation in alginate hydrogels the cells resumed normal metabolic activity (Figure 6.9 and 6.12). The maintenance of a low cell number throughout the initial few weeks of culture and the reduced metabolic activity of the individual cells that were present should allow for the infiltration of blood vessels into the TE structure to maintain cell viability and support subsequent tissue development after scaffold degradation *in vivo*.

### 6.3. Conclusion

NIH 3T3 fibroblasts encapsulated in either 2% or 5% w/v alginate remained viable for at least 150 days *in vitro* and were effectively mitotically inhibited in the initial five weeks culture. Normal cell proliferation in monolayer culture was observed after release of fibroblasts from the alginate hydrogel scaffolds. The reversible mitotic activity was mirrored by reversible partial catabolic inhibition. The growth and catabolic activity of fibroblasts was not influenced by the presence of the alginate hydrogel, treatment in calcium chloride or tri-sodium citrate. This indicated that the reversible growth and metabolic inhibition was due to the reversible mechanical confinement of cells. The maintained viability and reversible mitotic and catabolic inhibition of fibroblasts by encapsulation as dispersed entities in 2% and 5% w/v alginate hydrogels makes these constructs potentially suitable dermal grafts. Since the fibroblasts remain viable but are mitotically inhibited, they should initially act as a feeder layer to support keratinocytes to form the epidermis during the initial few weeks after implantation. Then, as the alginate hydrogel degrades, the fibroblasts should facilitate replacement of the dermal layer. Furthermore, since the alginate itself does not inhibit the growth or catabolism of cells which are not encapsulated it should allow uninhibited growth of keratinocytes co-transplanted on the surface of the graft to restore of the epidermis at the wound site.

# 7. Replacement of alginate hydrogel scaffold with normal ECM

In order that the tissue engineering approach is successful the rate of tissue growth should ideally occur at the rate of scaffold degradation (Mann *et al*. 2001b). It is therefore important that the scaffold remains *in situ* for sufficient time to support new tissue formation (Alsberg *et al*. 2003). Calcium cross-links are known to dissipate from Ca-alginate hydrogels over time during both *in vivo* and *in vitro* culture as a result of exposure of the hydrogels to ions such as sodium, potassium, magnesium and phosphate which cause the scaffold to degrade both *in vitro* and *in vivo* (Donati *et al.* 2009; Ikada 2006; Smidsrod & Skjakbraek 1990). The effect that encapsulated mammalian cells have on the degradation of alginate hydrogel is largely unknown.

Scaffold degradation results in changes in mechanical properties of hydrogel scaffolds (Boontheekul *et al.* 2005). Mechanical properties of scaffolds have been shown to impact on the cell proliferation, cell phenotype, growth factor production, ECM deposition and tissue development (Boontheekul *et al*. 2007; Bryant *et al.* 2004; Discher *et al.* 2005). Assessing the change in mechanical properties of scaffolds during culture is therefore important in determining the efficacy of a hydrogel as a cell encapsulating medium. The mechanical properties of hydrogels can be measured in a number of ways including compression and tensile testing (Boontheekul *et al.* 2005; Kong *et al*. 2004). When testing in compression, the sample's cross sectional area increases during the test and so it is difficult to obtain meaningful values for modulus and failure. While testing in tension gives less disputable results and gross mechanical deformation has been used previously to measure the tensile strength and modulus of alginate gels in tissue engineering (Wang *et al.* 2003), this method is subject to high variability which can be attributed to difficulties associated with clamping the sample.

Small deformation oscillatory rheology was used to measure changes in alginate hydrogel mechanical properties over time. Data collected using this technique can be analysed to precisely identify and discriminate between samples that are true gels (viscoelastic solids) or are concentrated polymer solutions which may appear gel like, but are actually viscoelastic liquids (Clark & Ross-Murphy 2009). The mechanical spectra of alginate hydrogel (acellular and cell-encapsulating) samples were characterized by measurement of the storage modulus ($G'$), loss modulus ($G''$) and complex viscosity ($\eta^*$) as a function of angular frequency, to

quantify the effect of culture media and cell presence on gel degradation over a period of 28 days *in vitro*. The changes in mechanical properties of FCM-incubated samples were also compared with changes in gel morphology, the release of calcium ions from the structure and changes in the wet and dry mass of the samples during culture.

The effect of gel degradation on the ECM production by the fibroblasts was also assessed. This was done by histological staining of fibroblasts encapsulated and released from both 2% and 5% w/v alginate hydrogels with HVG after three weeks encapsulation. This allowed for assessment of collagen expression, which is the main component of the ECM of dermis.

## 7.1. Results

### 7.1.1. Gel morphology

Visual evaluation of the acellular samples incubated in either water or FCM, and cell-encapsulating samples incubated in FCM was performed after 28 days culture (Figure 7.1). The images illustrate that all the hydrogels retained disc shape morphology despite the significant degradation of samples incubated in FCM, which was apparent when handling the samples. Acellular samples incubated in water showed no evidence of degradation upon handling. Furthermore, the dimensions of the samples were very similar to that at day 0 for all samples, with no evidence of gel contraction.

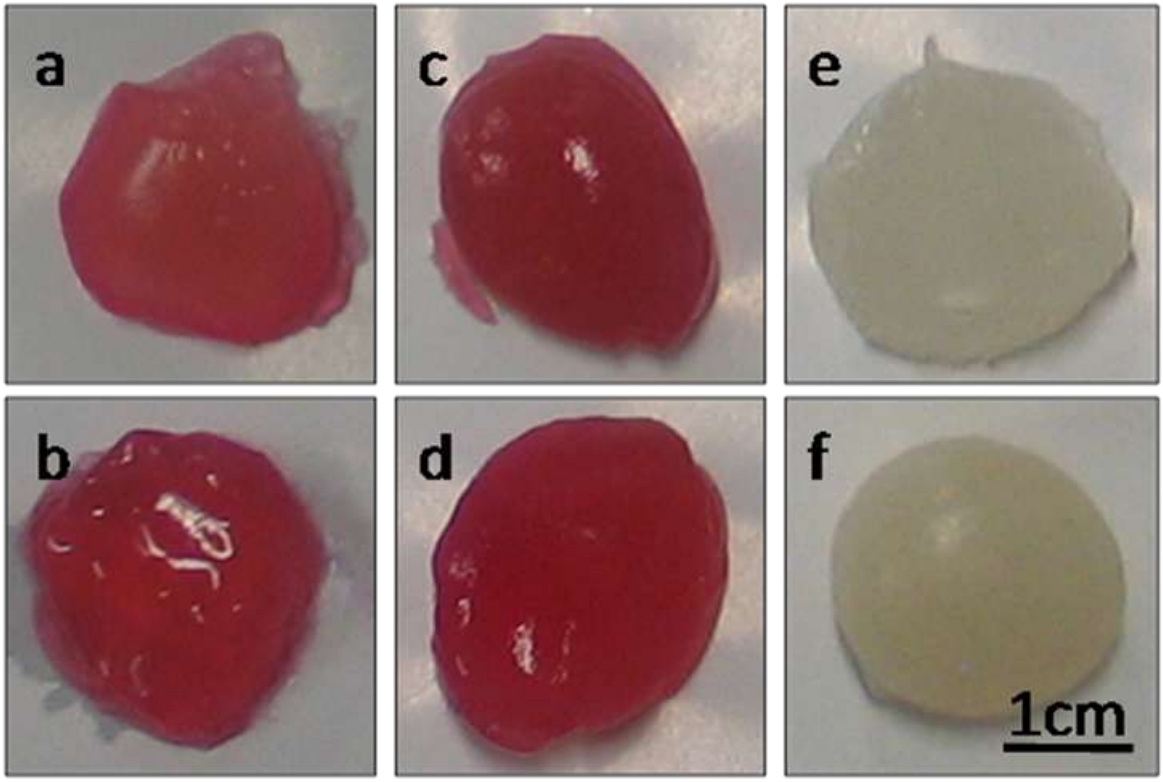



**Figure 7.1: Images of alginate gels at day 28. Samples incubated in growth media were a) 2% w/v acellular b) 2% w/v cellular c) 5% w/v acellular d) 5% w/v cellular. Samples incubated in water were e) 2% w/v acellular and f) 5% w/v acellular. The appearance of the gels illustrates that all samples retained a solid gel structure. The pink staining of the media incubated samples was due to the presence of phenol red pH indicator in the media. The photos illustrate that after 28 days all samples retained self-supporting structures of disc-shaped morphology, and retained dimensions similar to day 0. There was clear evidence of degradation in samples a-d but not in samples e-f.**

### 7.1.2. Rheology

Rheological measurements of G′, G″ and η* were performed to evaluate the impact of cell encapsulation and the effect of culture media on the mechanical properties of 2% and 5% w/v alginate gels incubated at 37°C for up to 28 days. Figure 7.2 shows the mechanical spectra for 5% and 2% w/v alginate samples at day one of the study. Predictably, the 5% w/v alginate samples gave higher values of G′ and G″ than the corresponding 2% w/v alginate samples. The spectra illustrate that all alginate samples showed true gel properties for at day one with G′>> G″ and no frequency dependence and a linear decrease in complex dynamic viscosity with increasing frequency.

Figure 7.3 shows measurements of (a) G′ and (b) G″ at 0.5% strain 1rad/sec for 5% w/v alginate samples over 28 day culture period. A dramatic reduction in G′ and G″ is observed within 7 days of culture in the alginate sample containing encapsulated 3T3 fibroblasts, with G′ falling to only 5% of its original value. A similar decrease in mechanical properties was also found with acellular alginate hydrogel, but the rate of reduction is much slower and at day 7 G′ for acellular samples were 30% the original value. By day 21, however, there was no significant difference ($p<0.05$) between the cellular and acellular samples. The alginate samples incubated in water showed no change over the study period as expected.

Similar trends were also witnessed in samples of 2% w/v alginate hydrogel (Figure 7.4) where G′ reduced in the cellular samples by 97% by day 7, whereas G′ of the acellular samples was found to be 40% of the original value and then reduced to 5% by day 21. As with the 5% w/v alginate samples there was no variation in G′ or G″ for the 2% w/v alginate samples incubated in water.

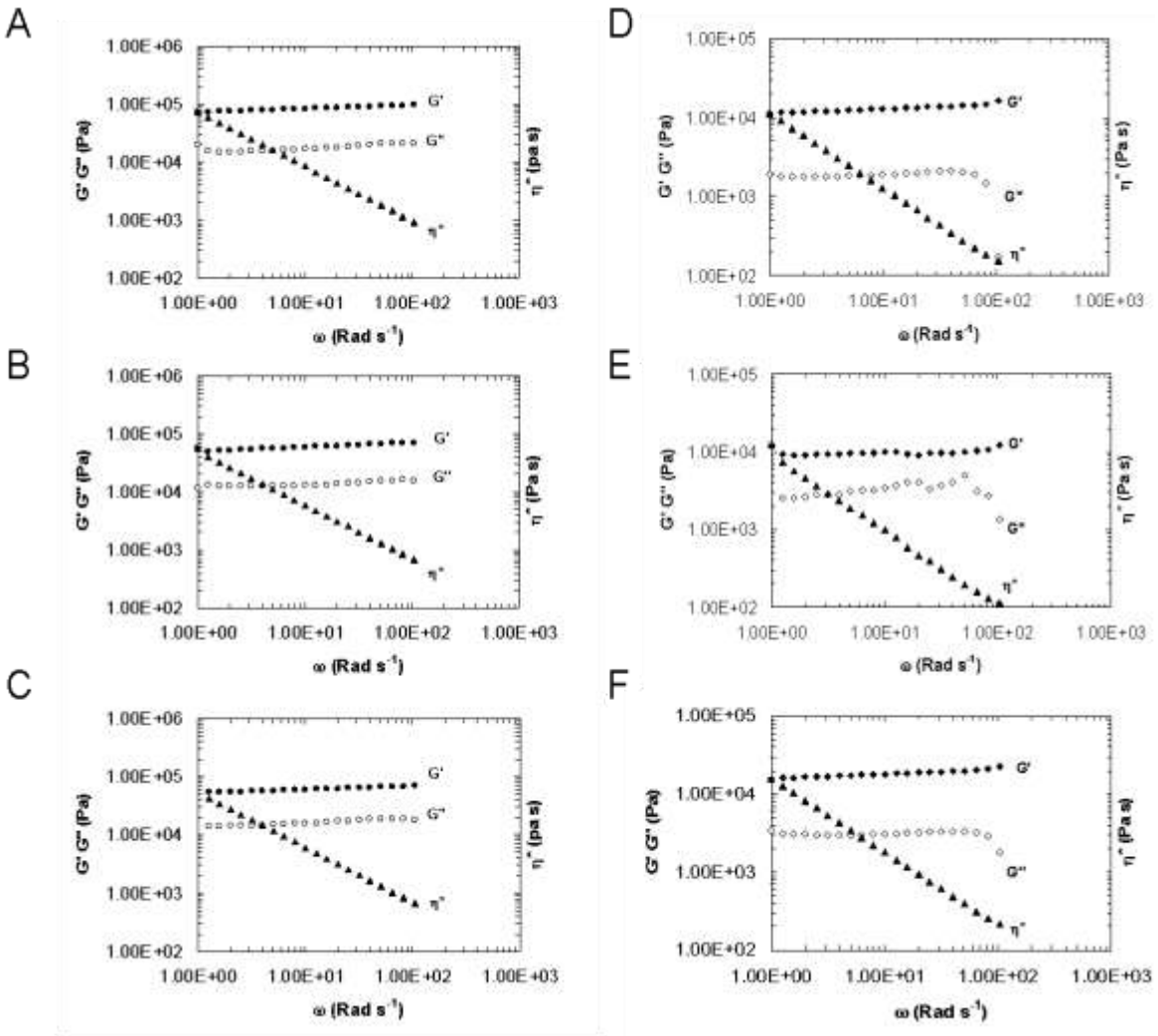
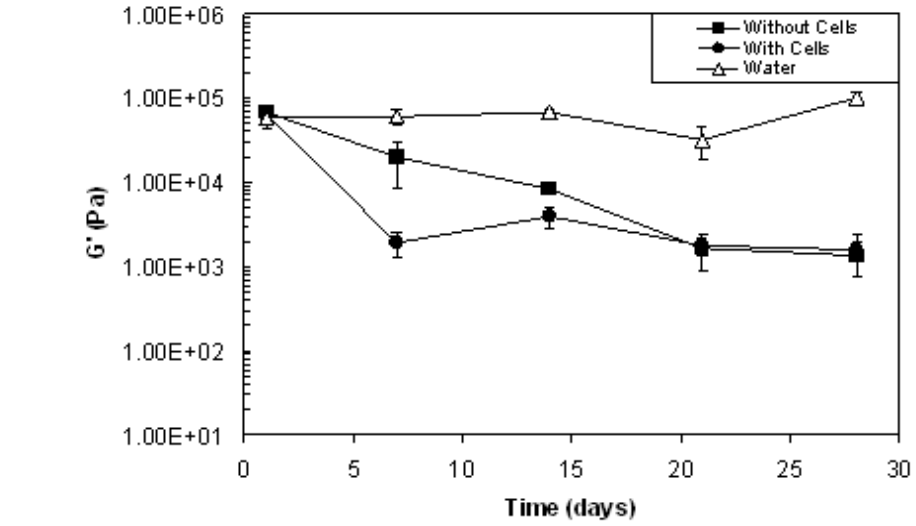
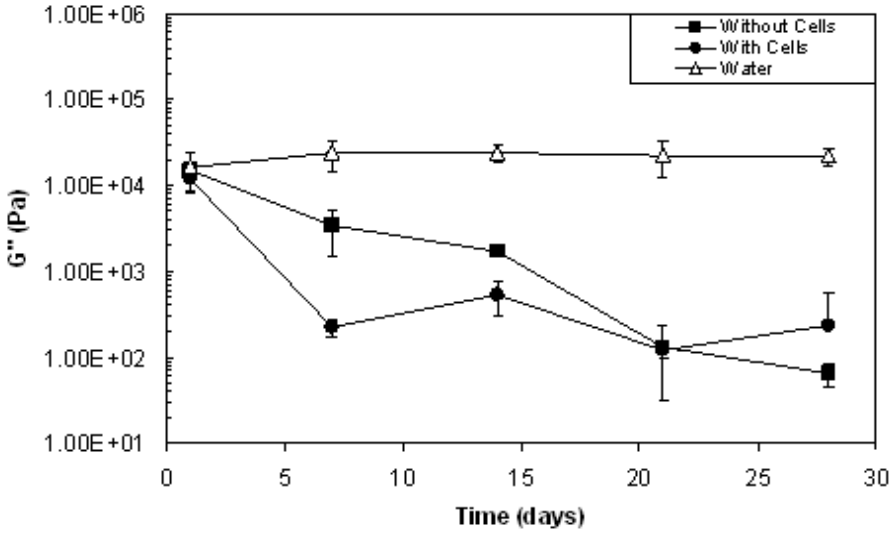



**Figure 7.2: Mechanical spectra (0.5% strain; 37°C), at day one showing variation of G′ (filled diamonds), G″ (open diamonds) and η* (filled triangles) with angular frequency for 5% w/v alginate hydrogel a) containing encapsulated 3T3 fibroblasts incubated in culture media, b) containing no cells incubated in culture media and c) containing no cells incubated in water, and 2% w/v alginate hydrogel d) containing encapsulated 3T3 fibroblasts incubated in culture media, e) containing no cells incubated in culture media and f) containing no cells incubated in water.**

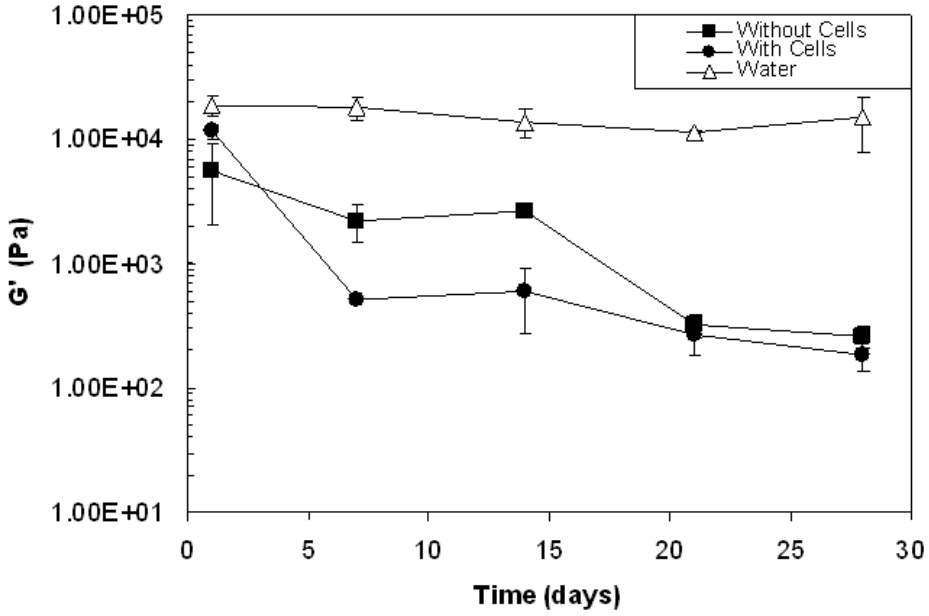



A)




B)

**Figure 7.3: Changes in a) G′ and b) G″ (6rad $s^{-1}$; 0.5% strain; 37°C), over 28 days incubation for 5% w/v alginate containing encapsulated 3T3 fibroblasts (filled circles), acellular alginate incubated in culture media (filled squares), and acellular alginate incubated in water (open triangles). Vertical error bars represent the standard deviation of the reported mean values (n=3). G′ and G″ for both media-incubated samples were significantly lower ($p<0.05$) than water-incubated samples at all time points, except day 1. G′ and G″ were significantly lower ($p<0.05$) for cell-encapsualting samples than acellular media-incuated samples at day 7.**



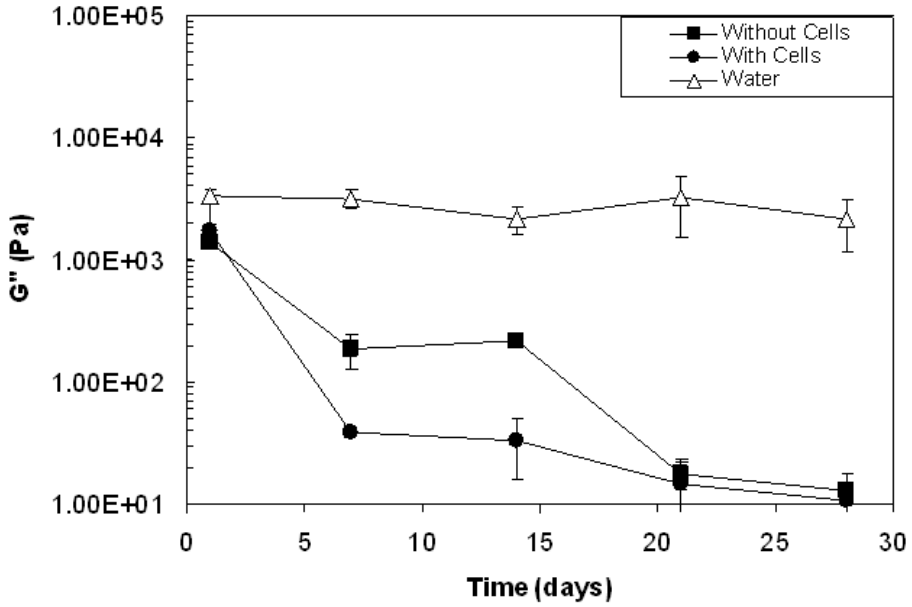




**Figure 7.4: Changes in a) G′ and b) G″ (6rad $s^{-1}$; 0.5% strain; 37°C), over 28 days incubation for 2% w/v alginate containing encapsulated 3T3 fibroblasts (filled circles), acellular alginate incubated in culture media (filled squares) and acellular alginate incubated in water (open triangles). Vertical error bars represent the standard deviation of the reported mean values (n=3). G′ and G″ for both media-incubated samples were significantly lower ($p<0.05$) than water-incubated samples at all time points, except day 1. G′ and G″ were significantly lower ($p<0.05$) for cell-encapsualting samples than acellular media-incuated samples at day 7 and 14.**

Mechanical spectra, however, shown in Figure 7.5 indicate that all the 5% w/v alginate samples show "true gel" properties (G′ >> G″) at day 28 but values of G′ and G″ are reduced by almost two decades compared with day one and at higher frequencies there is evidence of failure of the gels which does not occur at day one or in the sample incubated in water. The same trend is also observed in the 2% w/v alginate (Figure 7.7) samples, but failure of the gels occurs at lower frequency.

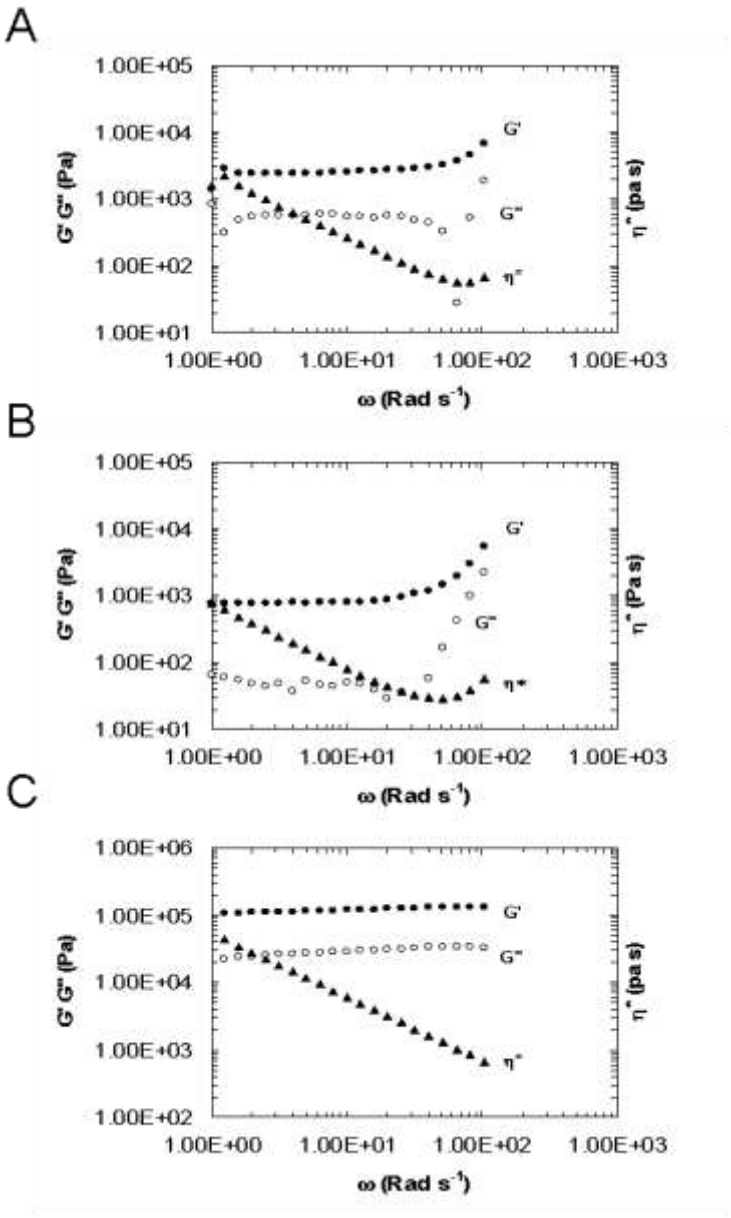



**Figure 7.5: Mechanical spectra (0.5% strain; 37°C), at day 28 showing variation of G′ (filled diamonds), G″ (open diamonds) and η* (filled triangles) with angular frequency for 5% w/v alginate a) containing encapsulated 3T3 fibroblasts incubated in culture media, b) containing no cells incubated in culture media and c) containing no cells incubated in water.**

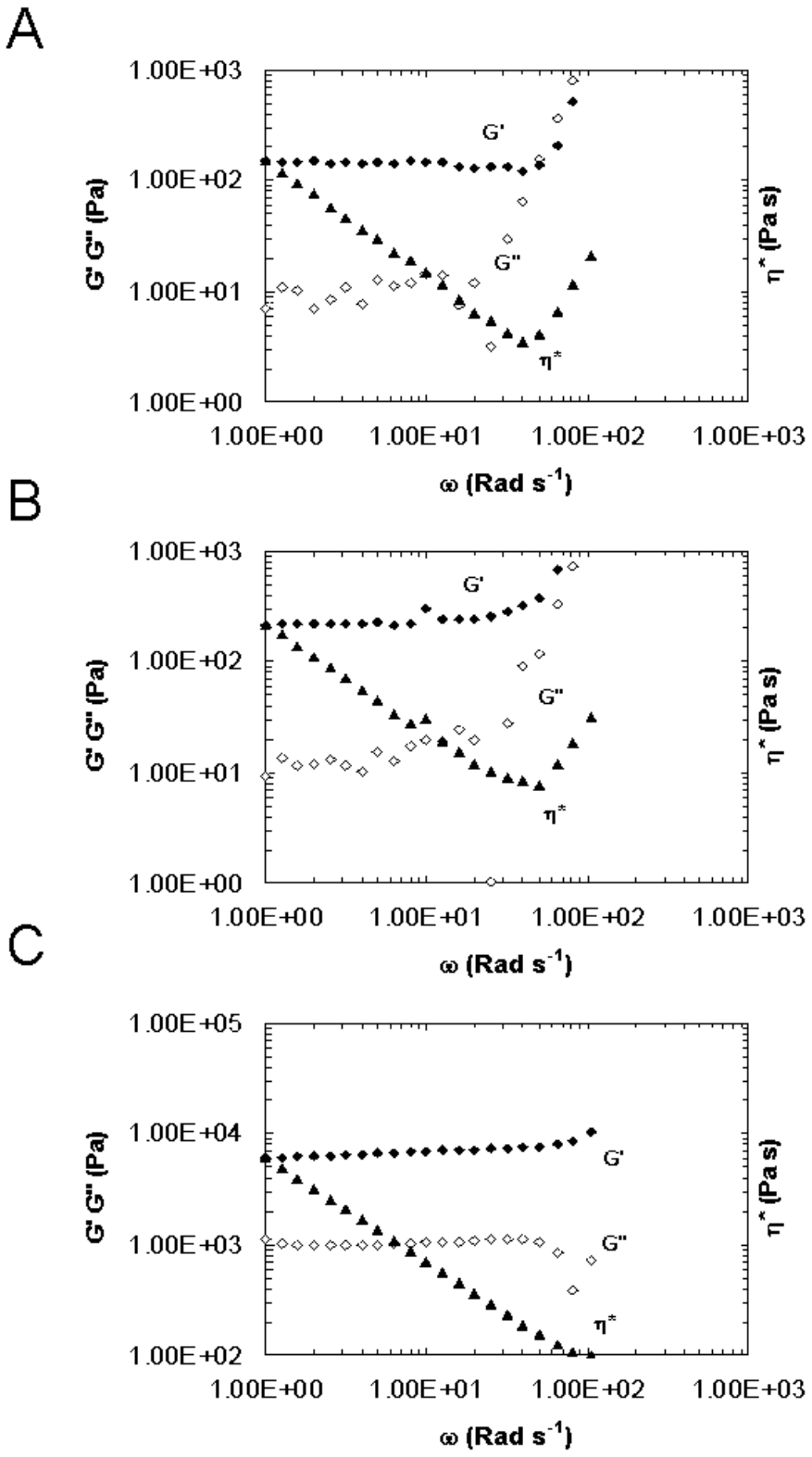



**Figure 7.6: Mechanical spectra (0.5% strain; 37°C), at day 28 showing variation of G′ (filled diamonds), G″ (open diamonds) and η* (filled triangles) with angular frequency for 2% w/v alginate a) containing encapsulated 3T3 fibroblasts incubated in culture media, b) containing no cells incubated in culture media and c) acellular incubated in water.**

### 7.1.3. Calcium release

The correlation of calcium ion release from constructs incubated in FCM with the changes in mechanical properties was assessed by measuring the change in calcium concentration of the media surrounding the alginates using ICP-MS (Figure 7.7). The release of calcium from both 2% and 5% w/v alginate does not appear to be affected by the presence of cells since the values at all time points are comparable between acellular and cell-encapsulating samples of the same concentration. For 2% w/v alginate hydrogel the greatest release of calcium into the media is seen within the first four days where the calcium concentration is around 7 times higher than at day 0, but at day six the calcium concentration of the media is still significantly higher ($P<0.05$) than at day 0. By day 11 the calcium concentration is not significantly different ($P>0.05$) from day 0 and after day 11 no significant changes in calcium concentration are observed. For 5% w/v alginate the release of calcium is seen to be highest in the first four days, where the calcium concentration becomes elevated by around 6.5 times that seen at day 0. The calcium concentration then falls between day four and 13 where the calcium concentration is not significantly different ($P>0.05$) from day 0. The changes in concentration of sodium, phosphate and potassium ion concentrations in the media over 25 days were also measured for all four samples (Figure 7.8). Concentrations of sodium, phosphate and potassium ions were seen to fluctuate throughout the culture period, but no clear correlation between changes in calcium concentration and the other ions were observed.

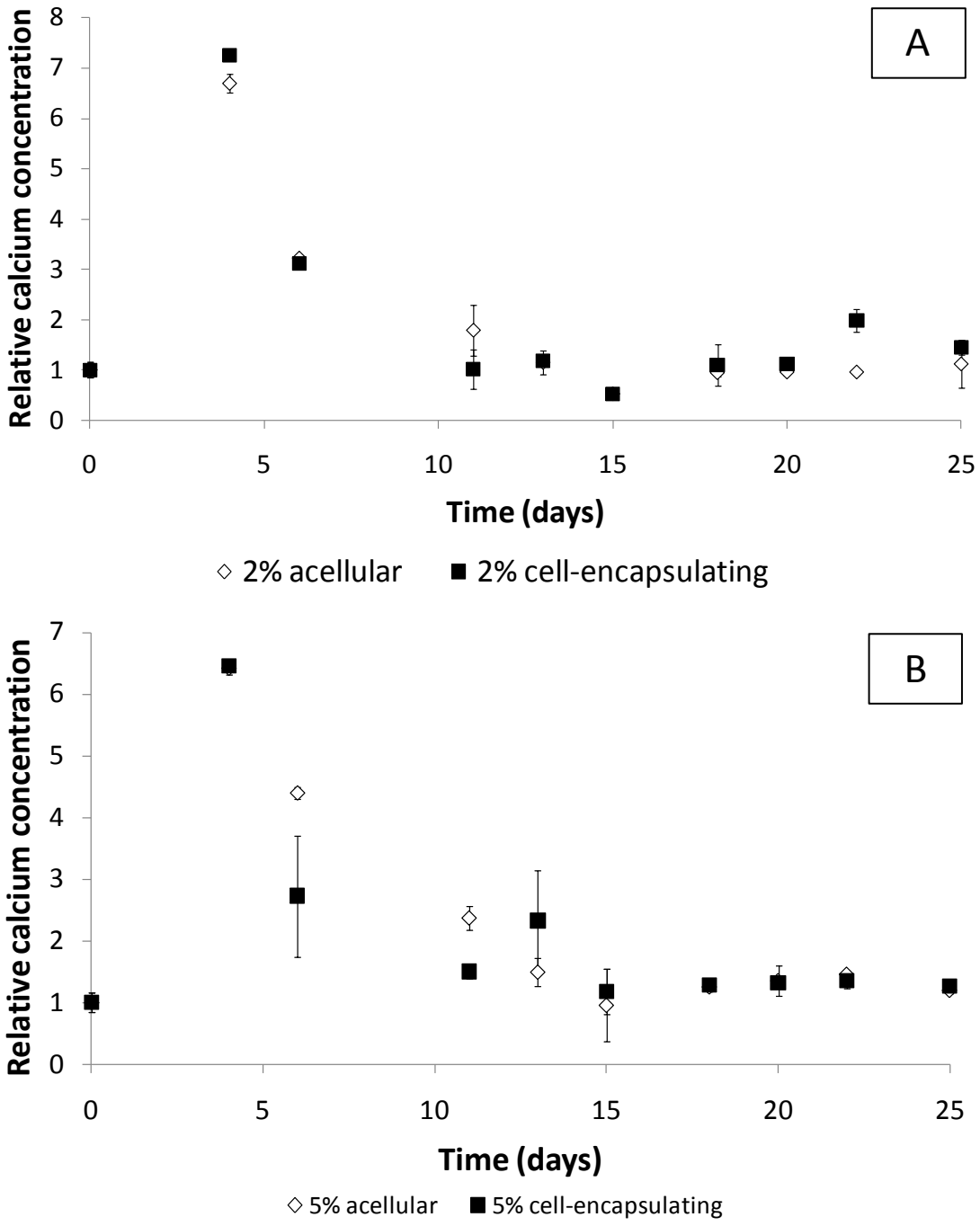



**Figure 7.7: Change in relative calcium concentration over 25 days culture of media surrounding a) 2% w/v acellular and cell-encapsulating and b) 5% w/v acellular and cell-encapsulating alginate hydrogel bead samples. Graphs show mean ± SD (n=3). The results show that in all samples the calcium release was greatest within the first four days of culture for all samples. Significant differences ($p<0.05$) between aceullar and cell-encapsulating hydrogels were only seen at day 11 for 5% w/v alginate hydrogels.**

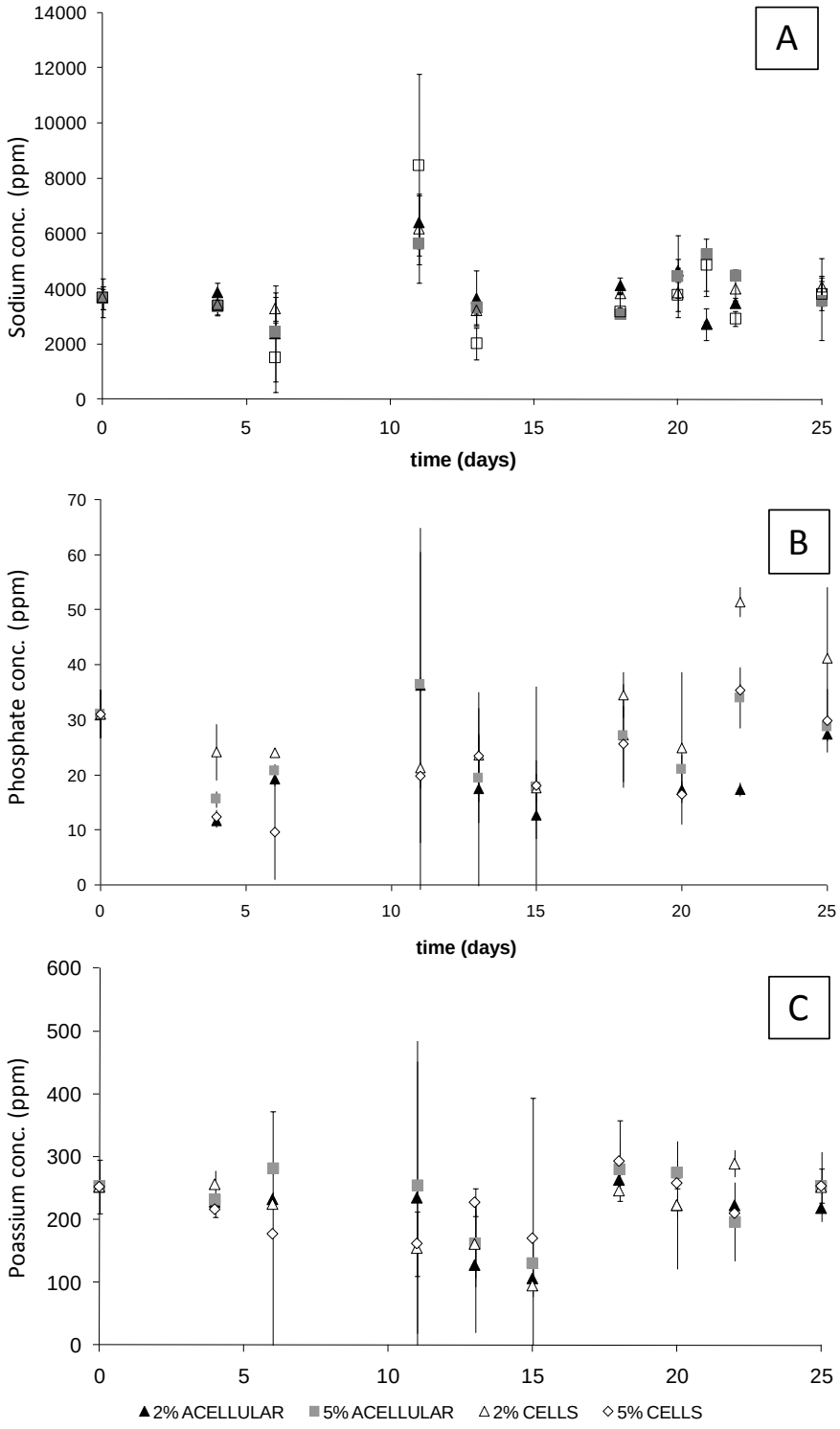



**Figure 7.8: Change in a) sodium ion b) phosphate ion and c) potassium ion concentration over 25 days culture of media surrounding 2% and 5% w/v acellular and cell-encapsulating alginate hydrogel bead samples. Graphs show mean ± SD (n=3). Fluctuations in all three ion concentrations was seen throughout the course of the experiment, but no clear correlation was observed.**

### 7.1.4. Change in hydrogel mass

The change in wet and dry mass of acellular and cell-encapsulating 2% and 5% w/v alginate hydrogel beads cultured in FCM were assessed over 33 days (Figures 7.9 and 7.10). The changes in hydrated and dry masses were very similar for corresponding acellular and cell-encapsulating hydrogels. The wet mass of 5% w/v hydrogels were seen to increase between day 0 and 18, and between day 0 and 14 for 2% w/v hydrogels. Thereafter, the wet mass was seen to gradually decrease until day 33 for all samples. The decrease in mass was seen to correlate with the formation of morphological defects such as cracks, and the breaking apart of beads.

The dry masses of 5% w/v cell-encapsulating and acellular alginate hydrogel were shown to decrease over 33 days, so that by day 33 around 80% of the original mass remained. It is evident that by reducing the alginate concentration to 2% w/v the rate of degradation was increased and that by day 33 only around 50% of the original alginate remained. The cells appeared to play very little part in this degradation, as with both 2% and 5% w/v alginate hydrogels, the degradation profiles are extremely similar for both acellular and cell-seeded samples. All four samples showed a significant decrease in dry mass between day 0 and 33 ($P<0.05$).

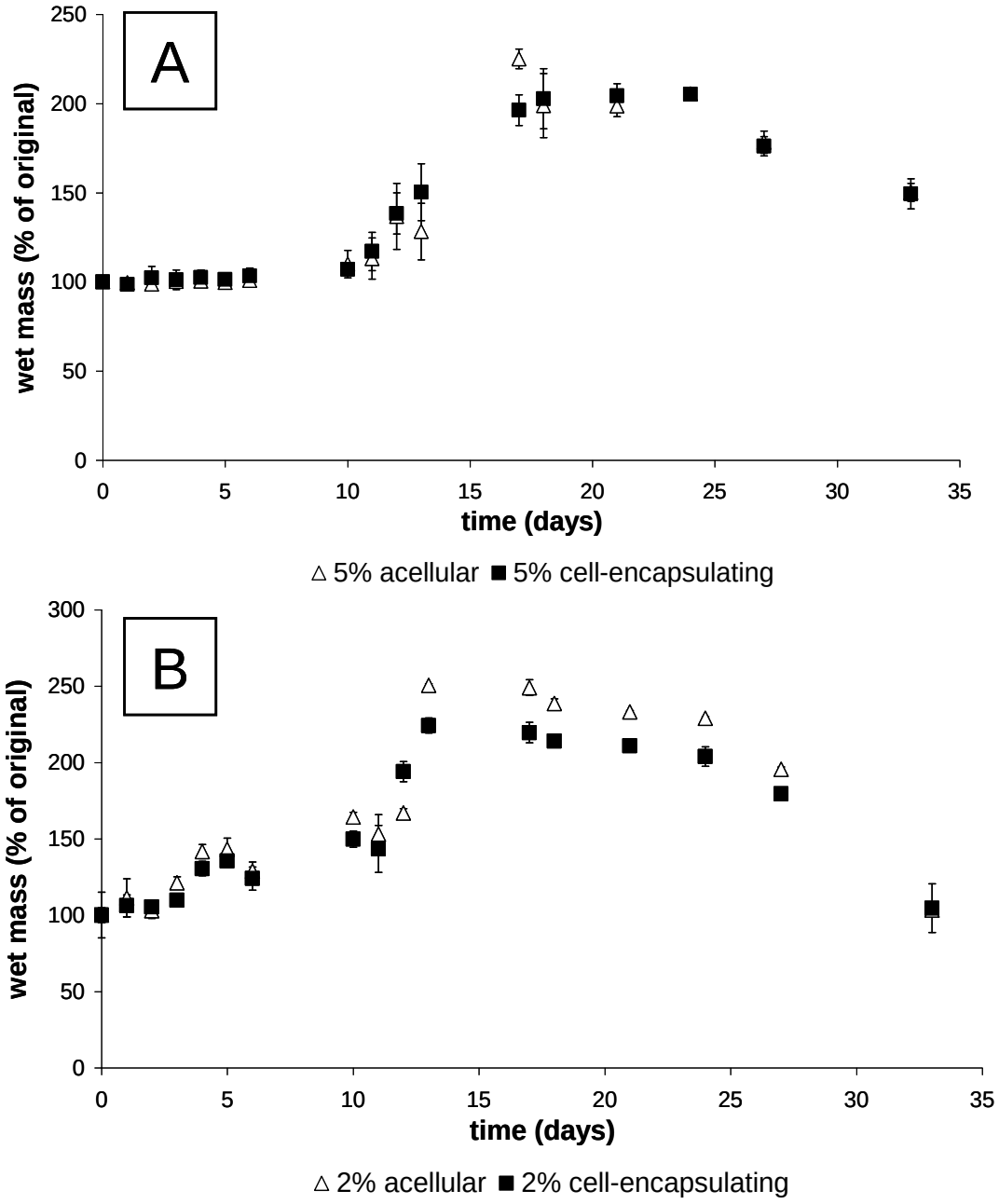



**Figure 7.9: Change in hydrated mass of a) 5% w/v and b) 2% w/v cell-encapsulating alginate hydrogel beads. Mean values ± SD are shown (n=3). The hydrated mass increased between day 0 and day 18 for 5% w/v alginate and between day 0 and 13 for 2% w/v alginate with alginate swelling then decreased as the alginate started to break apart and dissolve. No significant differences between acellular and cell-encapsualting samples were observed at any time opint for either 2% or % w/v alginate hydrogels.**

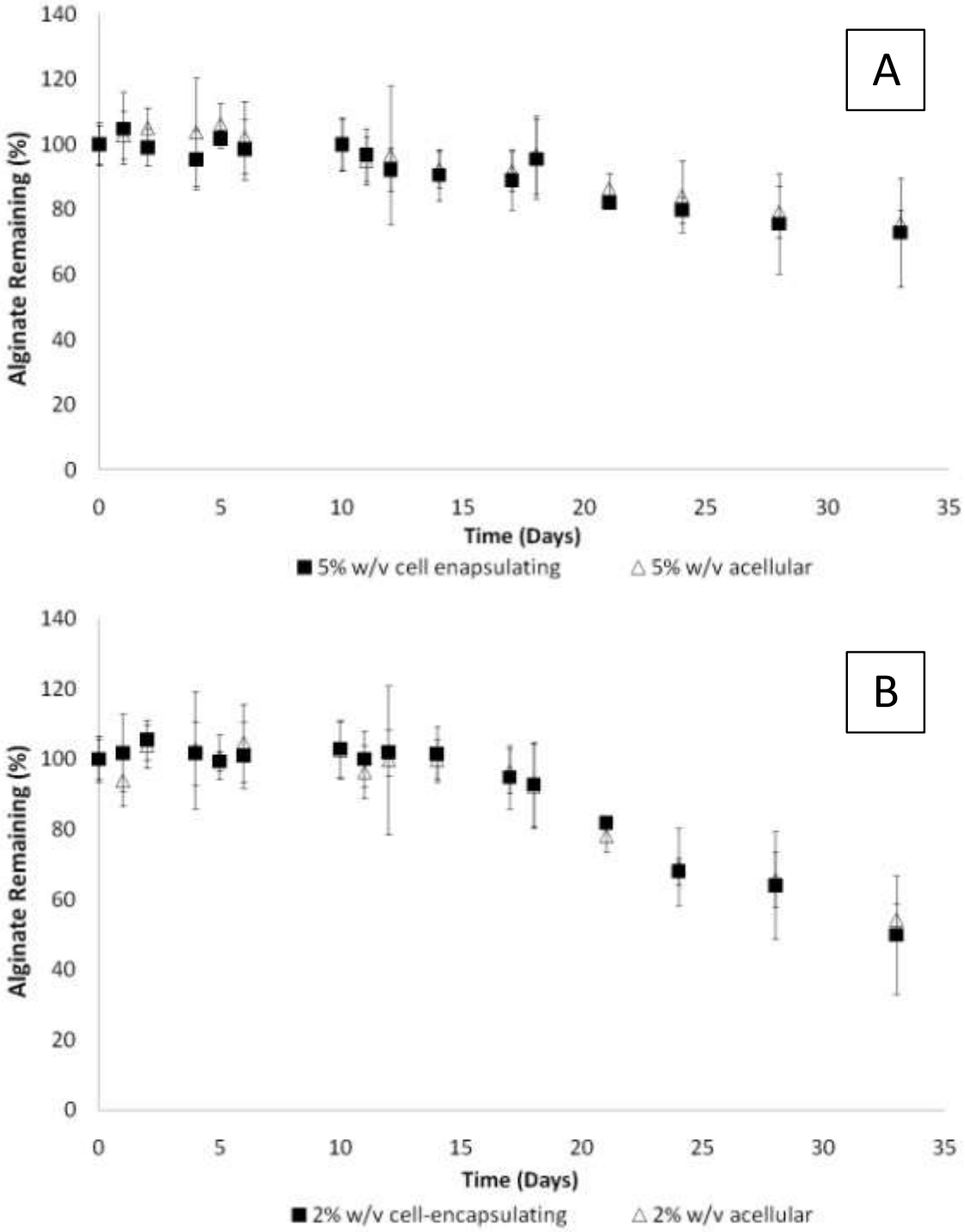



**Figure 7.10: Change in dry mass of of acellular and cell-seeded 5% and 2% w/v alginate hydrogel beads. Mean values ± SD are shown (n=3). Encapsulation of cells in the scaffolds had no appreciable effect on the degradation profile. It was also apparent that the 2% w/v alginate degrades faster than the 5% w/v alginate.**

### 7.1.5. Collagen production

To determine whether the encapsulated cells had deposited ECM histological sections of alginate encapsulated fibroblasts were stained with HVG after 21 days culture and compared with the normal dermis of rat skin (Figure 7.11). In Figure 11a the distribution of cells and collagen in the dermis of normal rat skin can be seen. The nuclei of cells stained blue-black due to the haematoxylin demonstrating the cell distribution, whilst the Van Gieson solution stains the collagen pink. It can be seen that the dermis has a low cell content and that the cells are present in small clusters or individual units and the main component of the dermis is collagen, which is present in small bundles of around 10-35μm diameter. The cells encapsulated within both 2% and 5% w/v alginate hydrogels (Figure 7.11b and 7.11c) remained as dispersed units within the hydrogel after 21 days of encapsulation and had not proliferated or migrated within the gel to form cell clusters as seen in the dermis of normal skin so the cell concentration was lower in the hydrogel than in normal rat skin. The haematoxylin also stained the alginate fibres in the hydrogel blue-black due to their negative charge and the positive charge of the haematoxylin. After 21 days there was no evidence of collagen presence in either 2% or 5% w/v alginate samples which indicated that the encapsulated fibroblasts failed to secrete ECM during this time period. It was, however, observed that as the alginate degraded the encapsulated fibroblasts were released from the alginate matrix and attached to suitable substrates such as glass or tissue culture plastic in which the alginate encapsulated cells were being cultured. Fibroblasts cultured on the glass slides for five days after release from both 2% and 5% w/v alginate hydrogels by degradation of the hydrogels in 100mM tri-sodium citrate were stained with HVG. The staining indicated that the fibroblasts were able to secrete collagen on the surface of the glass slide to which the cells had attached, at a level which appeared comparable to before they were encapsulated (Figure 7.12).

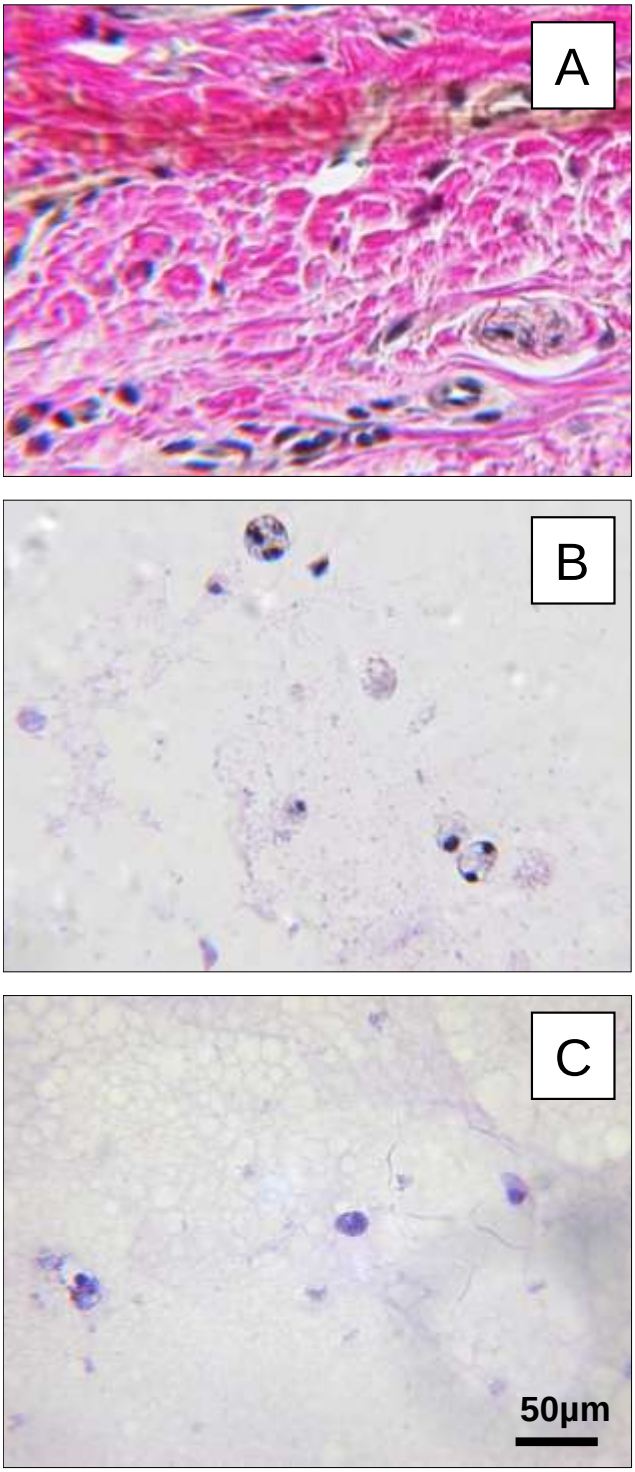



**Figure 7.11: HVG staining of normal rat dermis (A), and fibroblasts encapsulated in 2% w/v alginate (B) and 5% w/v alginate (C) after 21 days *in vitro* culture. The haemotoxylin stains the cell nuclei blue/ black and the Van Gieson Solution stains the collagen pink. In the dermis of skin there is a high collagen content, but after 21 days culture, there is no evidence of collagen presence within either 2% or 5% w/v alginate hydrogels.**

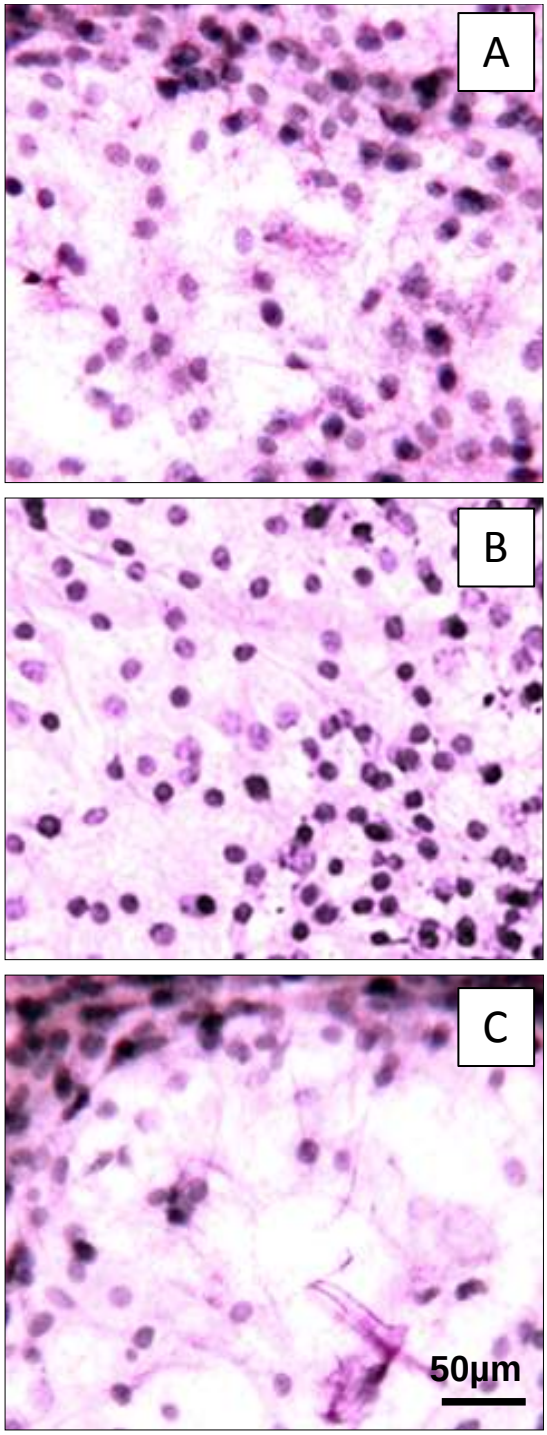



**Figure 7.12: HVG staining of 3T3 monolayers cultured on glass slides for five days a) before encapsulation, b) after release from 2% w/v alginate hydrogel after 21 days encapsulation and c) after release from 5% w/v alginate hydrogel after 21 days encapsulation. The Haematoxylin stains the cell nuclei blue/ black and the Van Gieson Solution stains the collagen pink. The staining shows that cells released from both 2% and 5% w/v alginate retain the ability to secrete collagen, as before encapsulation.**

## 7.2. Discussion

Alginate hydrogel is known to degrade over time by dissipation of calcium ions that cross-link the structure (Bajpai & Sharma 2004) as a result of exposure to monovalent cations such as sodium, potassium and phosphate which are present in cell culture media and the *in vivo* environment (Donati *et al.* 2009; Gombotz & Wee 1998; Ikada 2006; Smidsrod & Skjakbraek 1990; Sutherland 1991), and this is associated with a reduction in mechanical properties. Indeed, the mechanical properties of alginate hydrogels were seen to decrease with time in the presence of cell culture media but not in the presence of Millipore water, in which these ions are not present (Figures 7.3 and 7.4). The amount of calcium released from alginate hydrogels exposed to cell culture media was found to be very high within the first six days of culture for all alginates (Figure 7.7). This release of calcium ions was seen to correlate with a steep decline in the G′ and G" of these gels between day one and day 7 (Figures 7.3 and 7.4). This drastic decrease in mechanical properties has also been seen to occur in acellular Ca-alginate samples of 1-3% w/v in physiological saline during 7 days in culture conditions (Leroux *et al*. 1999). It has been suggested that exchange of ions and reordering of the gelled network occurs within the first 7 days and stabilizes thereafter (Drury *et al.* 2004).

It was expected that the movement of sodium, potassium ion and phosphate ions into the alginates would show a sharp drop in concentrations of these ions during the first 7 days in correlation with the sharp increase in calcium ions. Since the concentration of these ions in the media surrounding alginate samples was not seen to decrease drastically with the increase in calcium ion concentration (Figure 7.8) it can be concluded that the release of calcium ions results from the presence of these ions rather than their movement into the gels. Furthermore, it is possible that other ions present in the culture media such as lactate and citrate (Gombotz & Wee 1998; Sutherland 1991) influence the release of calcium cross-links from the alginate. Alternatively, the significant rise in calcium concentration of the media in the initial six days of culture may be due in part to the presence of excess un-bound calcium within the hydrogel which diffuses out the gel during this time period.

The G′ and G″ were seen to decrease more drastically in the first 7 days when cells were encapsulated in both 2% and 5% w/v gels. The differences in mechanical properties between acellular and cell-encapsulating samples could not however be attributed to differences in the calcium release profiles between cell encapsulating and acellular samples. It is possible that some of the calcium ions remaining within the alginates are used by the cells (Moore &

Dedman 1982) and thus not available for cross-linking of the alginate. For example, calcium is utilised by cells in cell signalling (Berridge *et al.* 2000), cell proliferation and adhesion to culture substrates (Rouzaire-Dubois & Dubois 2004).

The increased reduction of mechanical properties of the alginate hydrogels as a result of encapsulated fibroblasts in the initial week of culture may be of great importance for tissue engineering skin. The alginate hydrogel/ fibroblast dermal analogue will be seeded with keratinocytes after 7 days *in vitro* culture then implanted at the wound site. 5% w/v alginate hydrogel is preferable to 2% w/v alginate hydrogel, since the baseline properties of the 5% w/v alginate hydrogel are higher and there is therefore a reduced risk of gel disintegration upon implantation.

Between day 7 and day 28 further reductions in the mechanical properties of the alginate hydrogels incubated in FCM were observed. After 28 days the mechanical properties of cell-encapsulating and acellular samples of the same concentration in media were not significantly different ($P>0.05$) (Figures 7.4 and 7.5) and both the cell-encapsulating alginate hydrogels and their acellular counterparts were seen to remain as self supporting gels with similar dimensions to those seen at day 0 (Figure 7.6), and were seen to maintain gel-like mechanical spectra (Figures 7.7 and 7.8). Approximately 80% of the alginate remained in the 5% w/v alginate after 33 days culture and around 50% in the 2% w/v alginate, as confirmed by analysis of dehydrated mass of alginate hydrogel samples (Figure 7.10). It has been shown that the presence of a scaffold material to fill the wound space results in reduced scar formation (Heimbach *et al.* 1988; Wang *et al.* 2004). Alginate hydrogel is, therefore a preferable scaffold material compared with collagen and fibrin based scaffold materials, since these materials show increased degradation and contraction compared with both 2% and 5% w/v alginate hydrogels (Chun *et al.* 2003; Souren *et al.* 1989). In addition, the use of 5% w/v alginate hydrogel may be preferable to 2% w/v alginate hydrogel, since it shows a slower degradation profile in terms of mass change with culture time up to 33 days (Figure 7.10).

The long term stability of the gels should allow the encapsulated cells to be retained at the desired implantation location until sufficient ECM has accumulated (Kim & Mooney 1998; Koh & Atala 2004). Eventually, the alginate hydrogel implanted at a desired location should completely degrade and be resorbed (Smidsrod & Skjakbraek 1990) to leave behind new tissue which resembles the native host tissue. At three weeks encapsulation there was no

evidence of collagen presence within the alginate hydrogels (Figure 7.11) but when cells were released from the alginate hydrogel ECM production was evident (Figure 7.12). With the increased degradation that occurs with time increased ECM accumulation should occur, to facilitate tissue regeneration, as was seen with 1.95% w/v alginate hydrogel samples implanted into rats (Nunamaker *et al.* 2007). The inhibition of collagen production during encapsulation may have been due to the fibroblasts sensing the confinement of the 3D environment through cytoskeletal arrangement (Discher *et al.* 2005) which is known to alter cell behaviour in comparison with that exhibited in the 2D environment (Pedersen & Swartz 2005). This hypothesis is supported by the fact that in the body, fibroblasts only begin to secrete large quantities of ECM after injury (Welch *et al.* 1990), when the cells are released from the 3D confinement of the damaged ECM.

### 7.3. Conclusion

In this chapter it has been shown that alginate hydrogel may be a suitable scaffold material for the production of tissue engineered skin since it degrades relatively slowly over time in culture. This should allow for encapsulated fibroblasts to remain immobilised at the wound site upon implantation for extended periods where they will gradually be released from the scaffold as it degrades and secrete ECM to facilitate dermal repair. It has been demonstrated that 5% w/v alginate hydrogel may be preferable to 2% w/v alginate hydrogel since 5% w/v alginate hydrogel has higher baseline mechanical properties. The risk of gel disintegration upon implantation is therefore reduced if 5% w/v alginate hydrogel is used, compared with 2% w/v alginate hydrogel. Furthermore, since the dry mass of 5% w/v alginate hydrogel was seen to decrease less rapidly over time compared with 2% w/v alginate hydrogel, significantly more scaffold material should remain present at the wound site with time when 5% w/v alginate hydrogel is used for production of the dermal analogue. The alginate hydrogel acts as a filler material, and therefore, use of 5% w/v alginate hydrogel as the scaffold material may result in reduced scar formation compared with 2% w/v alginate hydrogel.

# 8. Angiogenesis

A problem with tissue engineered skin, as with other tissues, is clinical failure due to delays in vascularisation (Kaully *et al.* 2009; Sahota *et al.* 2004). It is generally accepted that the diffusion of nutrients and oxygen is limited to 100μm to 200μm (Rouwkema *et al.* 2008). Vascularisation is, therefore, essential to ensure adequate supply of nutrients to the transplanted cells. In addition, this vascular network acts as a transport system for hormones, waste products, and growth factors (Grellier *et al.* 2009). Inadequate vascularisation following implantation can result in cell death in tissue-engineered constructs (Rouwkema *et al.* 2008; Smith *et al.* 2004) and ineffective tissue development and integration (Malda *et al.* 2004; Yu *et al.* 2009).

Tissue engineering relies on angiogenesis for the vascularisation of new grafts (Cassell *et al.* 2002), which is the formation of new blood vessels by the growth and sprouting of existing vessels (Rouwkema *et al.* 2008). A range of growth factors and cytokines are known to be involved in angiogenesis, which stimulate different stages of blood vessel formation. The initiation of angiogenesis is known to be accelerated by the increased proliferation and migration of endothelial cells (ECs) (Bos *et al.* 2005; Egginton 2009) as a result of angiogenic factors such as VEGF (Bao *et al.* 2009; Keck *et al*. 1989; Leung *et al.* 1989; Noiri *et al.* 1998; Plouet *et al.* 1989; Yancopoulos *et al*. 2000; Yoshida *et al.* 1996), IL-6 (Fee *et al.* 2000; Jee *et al.* 2004; Nilsson *et al.* 2005; Yao *et al.* 2006), FGF-2 (Jansen *et al.* 2009; Yoshida, *et al.* 1996) and NGF (Cantarella *et al.* 2002; Emanueli *et al*. 2002; Nico *et al.* 2008; Zeng *et al.* 2010). VEGF is thought to be an especially important angiogenic factor (Ferrara 2009), and in addition to its mitogenic and chemotactic effect on ECs, it also increases vascular permeability to enhance angiogenesis (Ferrara 1999; Gavard & Gutkind 2006; Griffioen & Molema 2000; Senger *et al.* 1983; Senger *et al.* 1990; Yamagishi *et al.* 1999).

The direct application of VEGF to the wound site has been investigated as a possible approach to improve angiogenesis, but little success has been achieved (Lazarous *et al.* 1996), likely as a result of rapidly depleting and poorly controlled VEGF levels in the local tissue (Brem *et al.* 2009; Khurana *et al*. 2005). Sustained release of VEGF from grafts or dressings has been demonstrated to be more beneficial than direct application of VEGF. For example, VEGF has been incorporated into synthetic polymer scaffolds such as poly(lactide-

coglycolide) (PLG) (Chen *et al.* 2007), collagen scaffolds (Chiu & Radisic 2010) and alginate hydrogel beads (Gu *et al.* 2004) to provide sustained localised release. The sustained release of other angiogenic factors incorporated into scaffolds have also been shown to enhance angiogenesis (Moon & West 2008; Shi *et al.* 2008). Another approach to providing sustained release of growth factors is to incorporate cells which secrete angiogenic factors into the construct (Keshaw *et al.* 2005; Pinney *et al.* 2000).

Fibroblasts are known to produce angiogenic factors (Brenneisen *et al.* 2003; Detmar *et al.* 1997; Trompezinski *et al.* 2002; Tunyogi-Csapo *et al*. 2007), and the application of dermal fibroblasts to wound beds has been reported to accelerate angiogenesis (Chen *et al.* 2004). It was, therefore, hypothesised that fibroblasts encapsulated in alginate hydrogel scaffolds used in this thesis, may provide sustained release of angiogenic factors to promote vascularisation of the graft. In this chapter the transcription of genes encoding NGF, FGF-2, VEGF and IL-6 by fibroblasts following encapsulation ion 2% and 5% w/v alginate hydrogels was assessed. Since VEGF is of such importance in the stimulation of angiogenesis, the expression of VEGF protein by encapsulated cells was also assessed by ELISA, and compared with the level of expression seen in fibroblasts monolayer cultures.

To determine whether the angiogenic factors secreted by encapsulated fibroblasts were sufficient to induce angiogenesis, the recruitment and proliferation of ECs in the presence of alginate encapsulated fibroblasts was determined. This was compared with the proliferation and migration of ECs on transwell inserts in the presence of acellular samples of alginate hydrogels, or in plates which contained no alginate hydrogel or fibroblasts. These control samples allowed the effect of alginate hydrogel on proliferation and migration of endothelial cells to be determined.

## 8.1. Results

### 8.1.1. RT-PCR analysis of VEGF, FGF-2, NGF and IL-6 expression by fibroblasts

The potential of fibroblasts encapsulated in both 5% w/v and 2% w/v alginate hydrogels to induce vascularisation of the scaffolds was assessed by RT-PCR analysis of VEGF, FGF-2, IL-6 and NGF expression, compared with GAPDH expression (Figures 8.1 and 8.2). The expression of these genes was assessed at one and three weeks post encapsulation, while still encapsulated, or following release from encapsulation by degradation of the alginate hydrogel in citrate, followed by 48h of monolayer culture on tissue culture plastic. Control samples

were monolayer cultures of fibroblasts which had not been previously encapsulated in alginate hydrogel. Visual examination shows that VEGF and NGF were expressed by fibroblasts both during encapsulation and following release from 5% and 2% w/v alginate hydrogels. In contrast, FGF-2 transcription was not evident following encapsulation in 5% w/v alginate, and was only evident at week one encapsulation in 2% w/v alginate, and not in other conditions. The relative expression of VEGF, FGF-2, NGF and IL-6 according to band intensity were determined and are represented in Figures 8.3-8.6.

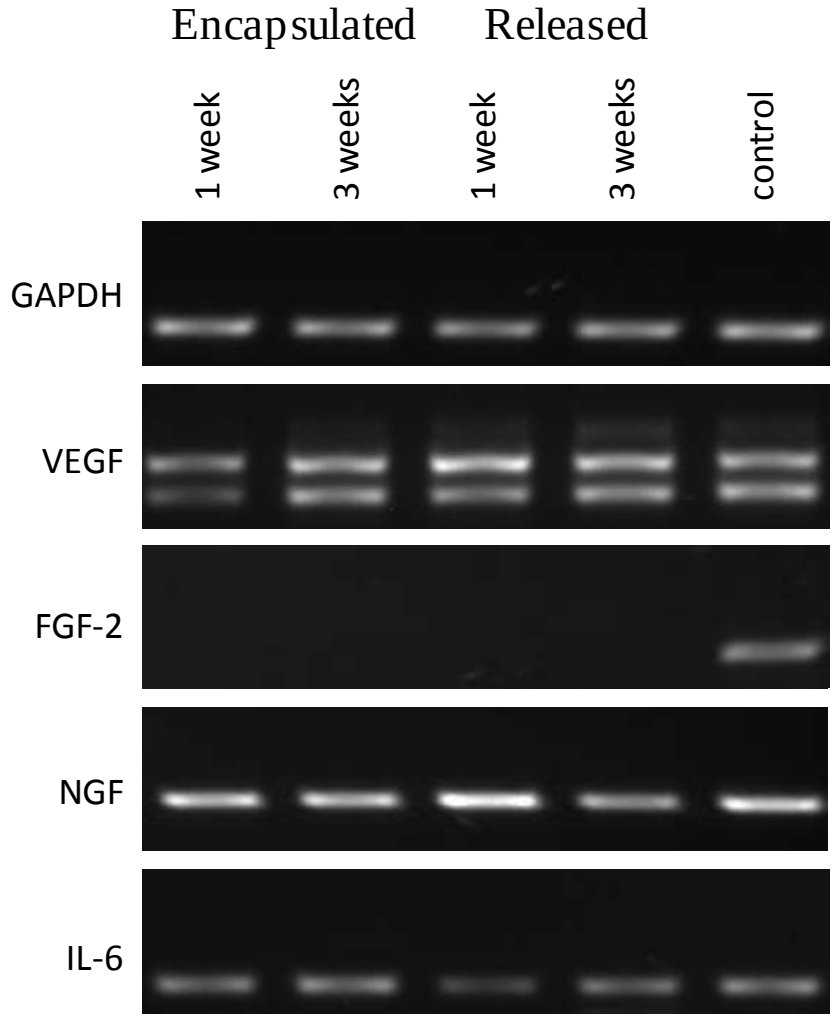



**Figure 8.1: RT-PCR analysis of GAPDH, VEGF, FGF-2, NGF and IL-6 expression by 3T3 fibroblasts following encapsulation in 5% w/v alginate for one or three weeks, compared with before encapsulation (control). Transcription of the genes was analysed either during encapsulation or following release from encapsulation and monolayer culture for 48h.**

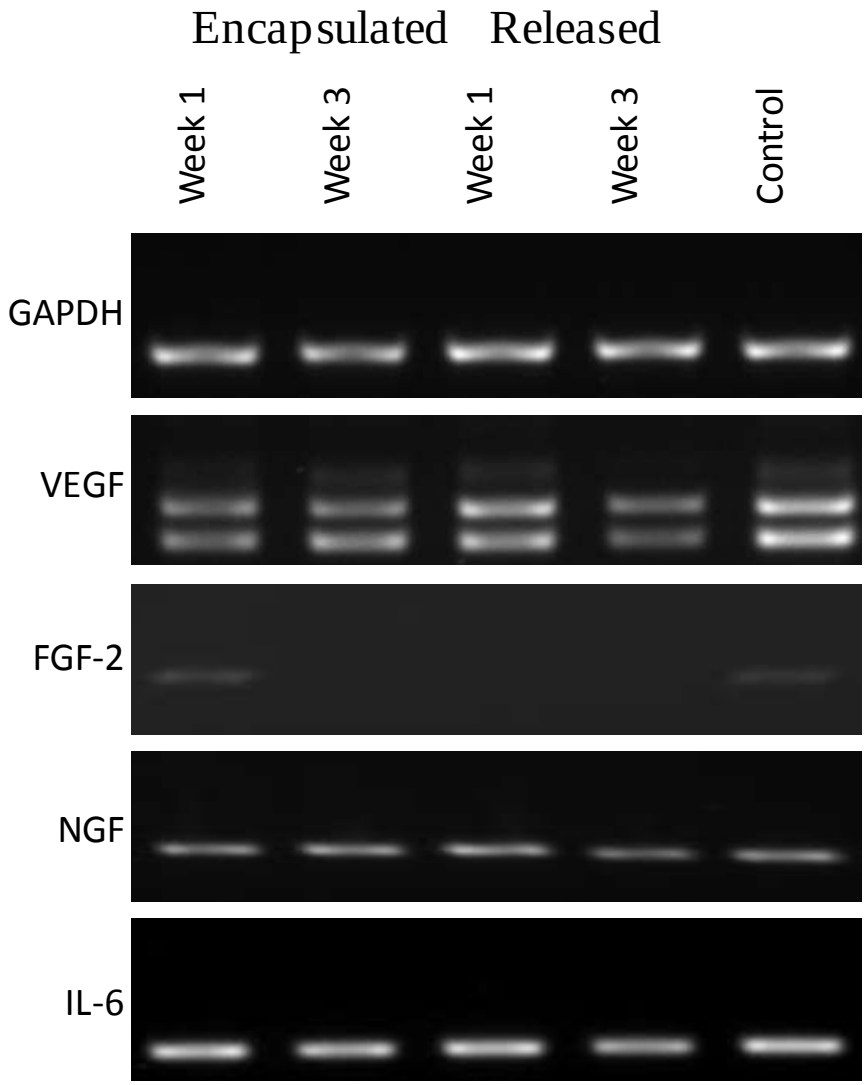



**Figure 8.2: RT-PCR analysis of GAPDH, VEGF, FGF-2, NGF and IL-6 expression by 3T3 fibroblasts following encapsulation in 2% w/v alginate for one or three weeks, compared with before encapsulation (control). Transcription of the genes was analysed either during encapsulation, or following release from encapsulation and monolayer culture for 48h.**

Variations in the expression of the three different VEGF isoforms were seen following encapsulation in 5% w/v alginate hydrogel (Figure 8.3a), but generally, VEGF transcription was down-regulated at week one following encapsulation, but was comparable with the control after three weeks encapsulation. After release from encapsulation at week one, the transcription of VEGF was seen to increase compared with during encapsulation, to a level comparable with the control. The transcription of VEGF at week three after release was also comparable with the control.

The expression of the three different VEGF isoforms by fibroblasts following encapsulation in 2% w/v alginate was at different levels in each condition (Figure 8.3b), as following encapsulation in 5% w/v alginate hydrogel (Figure 8.3a). The transcription of VEGF was down-regulated following one week encapsulation in 2% w/v compared with the control, as in 5% w/v alginate. This down-regulation was, however, less extensive than following encapsulation in 5% w/v alginate. The expression of VEGF increased after three weeks encapsulation compared with one week encapsulation, to a level comparable with the control, as was observed following encapsulation in 5% w/v alginate hydrogel. Following release from encapsulation in 2% w/v alginate hydrogel the expression of VEGF at week one was seen to be up-regulated compared with sustained encapsulation, to a level comparable with the control. At week three, however, the fibroblasts released from 2% w/v alginate showed reduced transcription of VEGF both compared with the control and the encapsulated samples at week three.

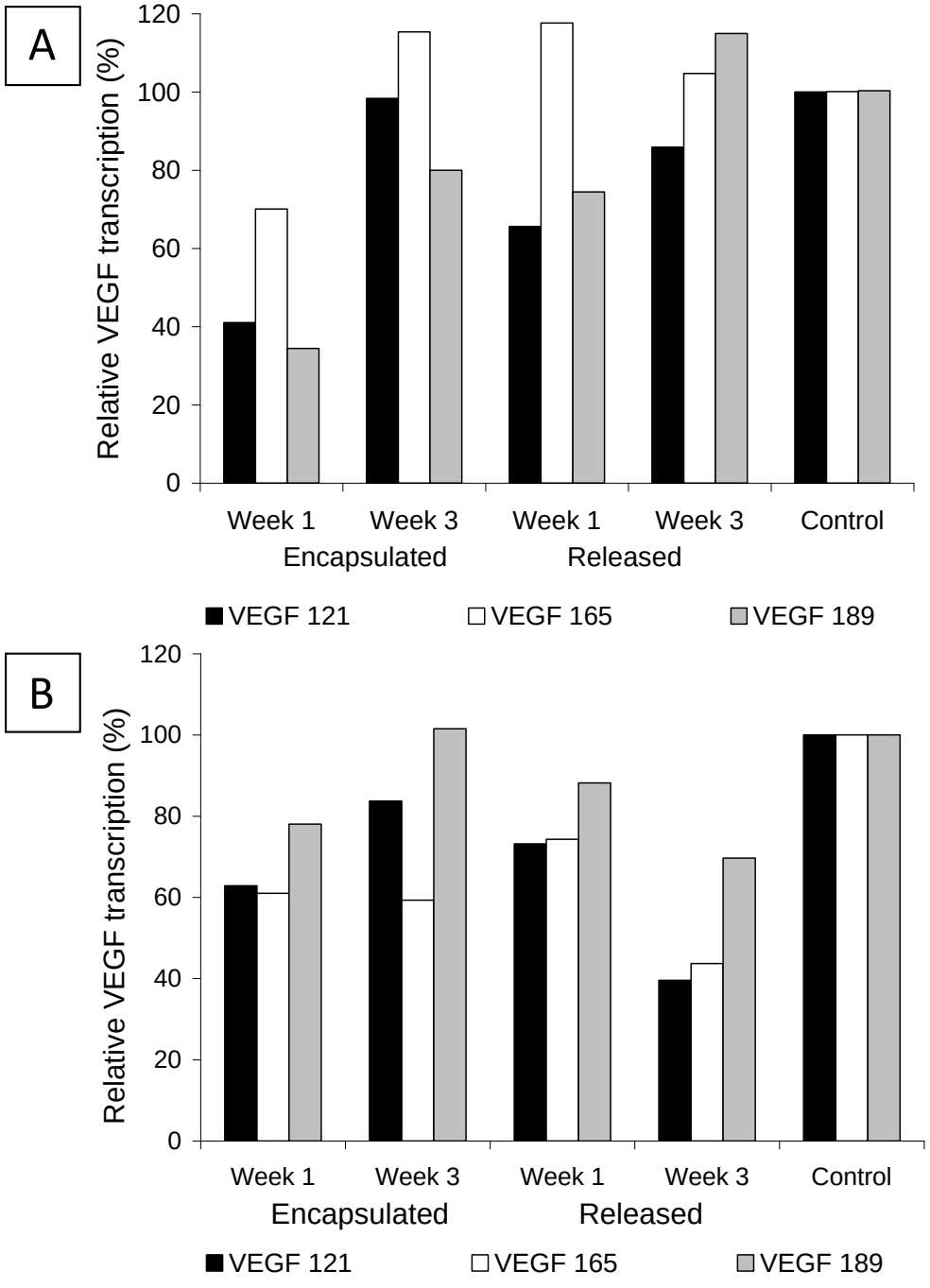



**Figure 8.3: Relative transcription of VEGF isoforms 121, 165 and 189 by fibroblasts following encapsulation in a) 5% w/v alginate hydrogel or b) 2% w/v alginate hydrogel, compared with before encapsulation (control). Transcription of VEGF was analysed either during encapsulation or following release from encapsulation and monolayer culture for 48h. Expression of all three VEGF isoforms is seen in all five conditions, although the level of expression varies with differing conditions.**

Quantification of band intensity revealed that FGF-2 transcription by fibroblasts encapsulated and released from 5% w/v alginate hydrogel was reduced to less than 10% of that seen before encapsulation (Figure 8.4a). This severe reduction in FGF-2 transcription was also observed at week three encapsulation and weeks one and three after release form encapsulation in 2% w/v alginate hydrogel (Figure 8.4b). The expression of FGF-2 at week one encapsulation in 2% w/v alginate was, however, comparable with the control.

Following encapsulation in 5% w/v alginate, the transcription of NGF was seen to be reduced to approximately 35% and 40% of that seen before encapsulation at week one and week three, respectively. Following release from encapsulation, the level of NGF expression was seen to more than double after one week encapsulation, to approximately 75% of that seen in the control sample. After three weeks encapsulation the level of NGF expression was only seen to be approximately 30% of that seen in the control (Figure 8.5a). The transcription of NGF by fibroblasts encapsulated in 2% w/v alginate was seen to be comparable with the control at week one encapsulation, and was seen to be up-regulated compared with the control at three weeks encapsulation. Following release from 2% w/v alginate, the level of NGF transcription at one week was seen to be approximately 10% higher than the control, and at three weeks, approximately 15% lower than the control (Figure 8.5b).

IL-6 transcription during encapsulation in 5% w/v alginate hydrogel was comparable with the control at week one and was up-regulated when compared with the control at week three. Following release from encapsulation, the fibroblasts continued transcribing IL-6, but at week one, this expression was down-regulated by ~50% compared with the control. Transcription of IL-6 at week three following release was, however, at a level comparable with the control (Figure 8.6a). In comparison, the expression of IL-6 by fibroblasts following encapsulation in 2% w/v alginate was seen to fluctuate by only ~15% compared with the control (Figure 8.6b).

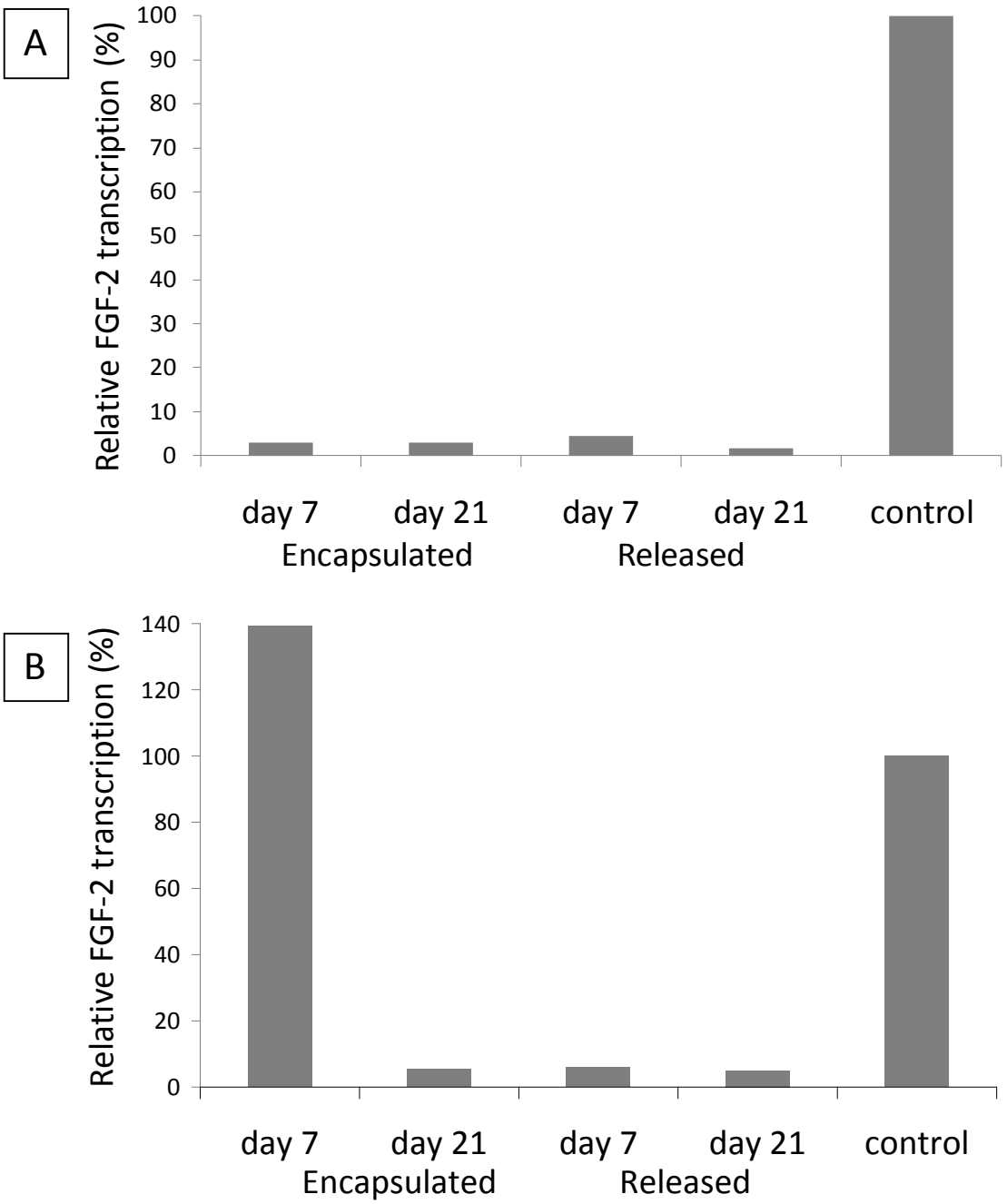



**Figure 8.4: Relative transcription of FGF-2 by fibroblasts following encapsulation in a) 5% w/v alginate hydrogel or b) 2% w/v alginate hydrogel, compared with before encapsulation (control). Transcription of FGF-2 was analysed either during encapsulation or following release from encapsulation and monolayer culture for 48h. Expression of FGF-2 was reduced to less than 5% of that seen before encapsulation both during encapsulation and after release from 5% w/v alginate hydrogel. Extensive down-regulation of FGF-2 transcription was also observed following encapsulation in 2% w/v alginate hydrogel, although to a lesser degree.**

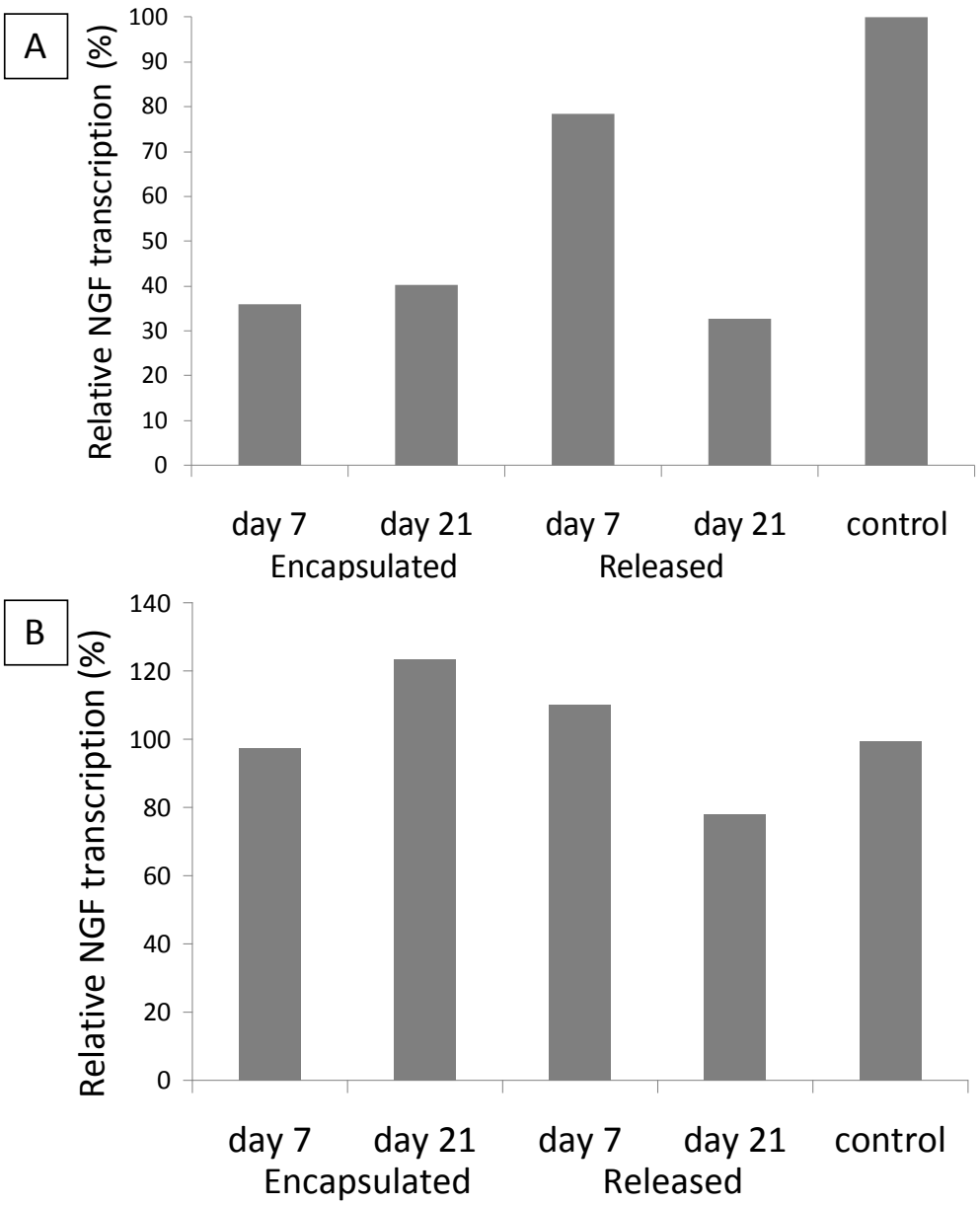



**Figure 8.5: Relative transcription of NGF by fibroblasts following encapsulation in a) 5% w/v alginate hydrogel or b) 2% w/v alginate hydrogel, compared with before encapsulation (control). Transcription of NGF was analysed either during encapsulation or following release from encapsulation and monolayer culture for 48h. Expression of NGF was observed to be maintained following encapsulation in both 5% and 2% w/v alginate hydrogel. Transcription during encapsulation in 5% w/v alginate was down-regulated compared with the control, which was reversed after release at one week, but not after release at three weeks. Transcription of NGF was less effected by encapsulation in 2% w/v alginate hydrogel.**

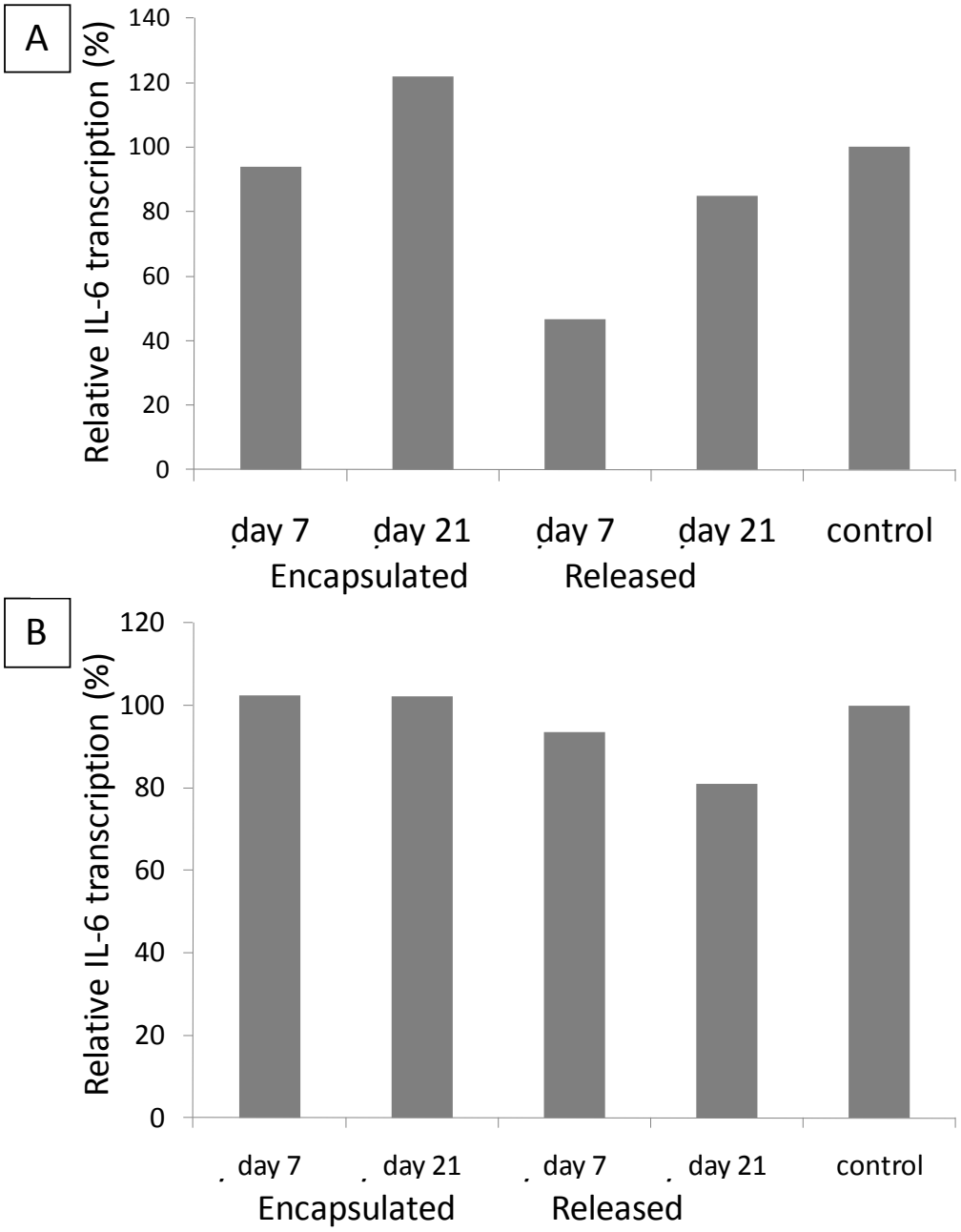



**Figure 8.6: Relative transcription of IL-6 by fibroblasts following encapsulation in a) 5% w/v alginate hydrogel or b) 2% w/v alginate hydrogel, compared with before encapsulation (control). Transcription of IL-6 was analysed either during encapsulation or following release from encapsulation and monolayer culture for 48h. Expression of IL-6 was observed to be maintained following encapsulation in both 5% and 2% w/v alginate hydrogel, although a ~50% down-regulation of transcription was evident at day 7 after release from encapsulation. In comparison, the fluctuation in IL-6 transcription following encapsulation in 2% w/v alginate was seen to only be ~15%.**

### 8.1.2. VEGF ELISA

Since VEGF is thought to be the main cell signalling molecule involved in the induction of angiogenesis, the level of VEGF protein expression by encapsulated fibroblasts was assessed by ELISA, and compared with VEGF expression by fibroblast monolayers. Figure 8.7 shows the expression of VEGF by fibroblasts encapsulated in 2% and 5% w/v alginate hydrogels up to three weeks post-encapsulation. The level of VEGF expression remained relatively constant throughout the course of the study, and a million encapsulated cells secreted approximately 1400pg of VEGF within the three days prior to sampling at all time points for both 2% and 5% w/v encapsulated fibroblasts. The amount of VEGF protein expressed by monolayer cultures of fibroblasts before encapsulation was also assessed by VEGF ELISA and found to be 5481±588.5pg/million cells. This demonstrated that VEGF protein expression by encapsulated fibroblasts was reduced by approximately 75% compared with before encapsulation.

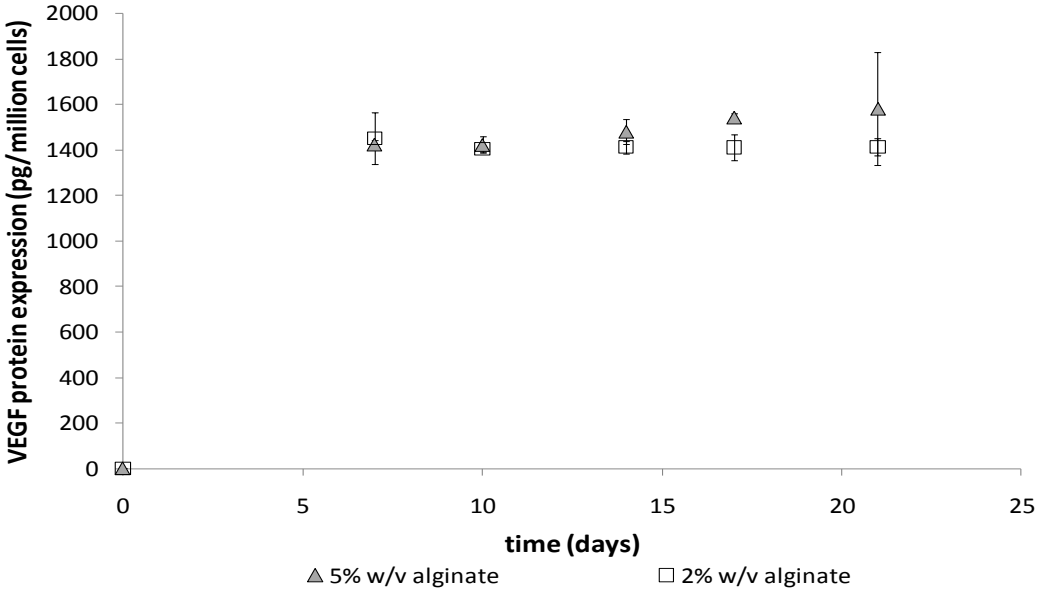



**Figure 8.7: Amount of VEGF protein secreted by fibroblasts encapsulated in 2% or 5% w/v alginate hydrogels as determined by VEGF ELISA up to 21 days post-encapsulation. Mean values ± SD are shown (n=3). The expression of VEGF protein was seen to be constant throughout the course of the study at approximately 1400pg/ million fibroblasts for both 2% and 5% w/v alginate encapsulated fibroblasts. The expression of VEGF protein was seen to be significantly ($p<0.05$) reduced by encapsulation in alginate since before encapsulation, fibroblasts monolayers were seen to express 5481±588.5pg VEGF/ million cells. No significant differences ($p<0.05$) between the amount of VEGF produced by 2% and 5% w/v encapsulated cells were seen at any time point.**

### 8.1.3. EC migration and proliferation

In order to assess whether fibroblasts encapsulated in 5% and 2% w/v alginate hydrogel would induce angiogenesis, the migration and proliferation of ECs co-cultured with the encapsulated fibroblasts were assessed and compared with control samples of acellular 5% and 2% w/v alginate hydrogels, respectively. Figure 8.8 shows the growth of ECs in the presence of acellular and fibroblast-encapsulating samples of 5% w/v and 2% w/v alginate hydrogels. It was evident that proliferation of ECs occurred in the presence of all four alginate hydrogel samples and the growth of ECs was not affected by the presence of encapsulated fibroblasts in either 5% or 2% w/v alginate hydrogels. Figure 8.9 shows the number of ECs that migrated toward 5% and 2% w/v fibroblast-encapsulating and acellular alginate hydrogels with time. It was evident that ECs migrated toward all four samples, and that the presence of fibroblasts within both 5% and 2% w/v alginate had no significant effect on the rate of migration.

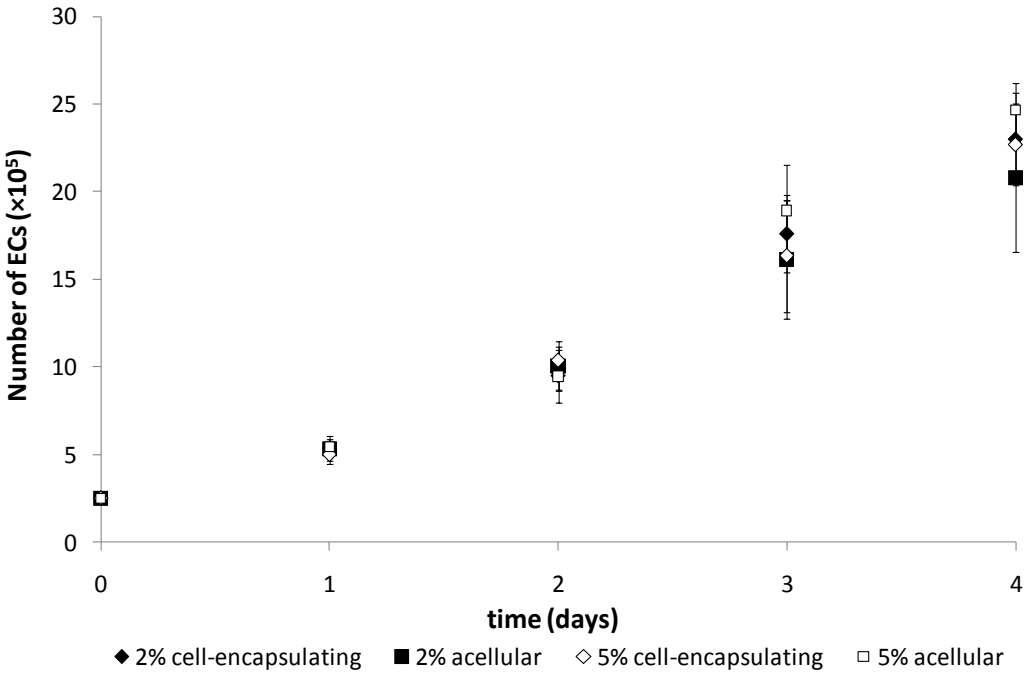



**Figure 8.8: Number of viable ECs present on membrane supports up to four days culture in the presence of acellular and fibroblast-encapsulating samples of 2% and 5% w/v alginate hydrogels. Mean values ± SD are shown (n=3). Presence of fibroblasts within the alginate hydrogels, or changing the alginate hydrgogel concentration had no significant ($p>0.05$) effect on the proliferation of ECs.**

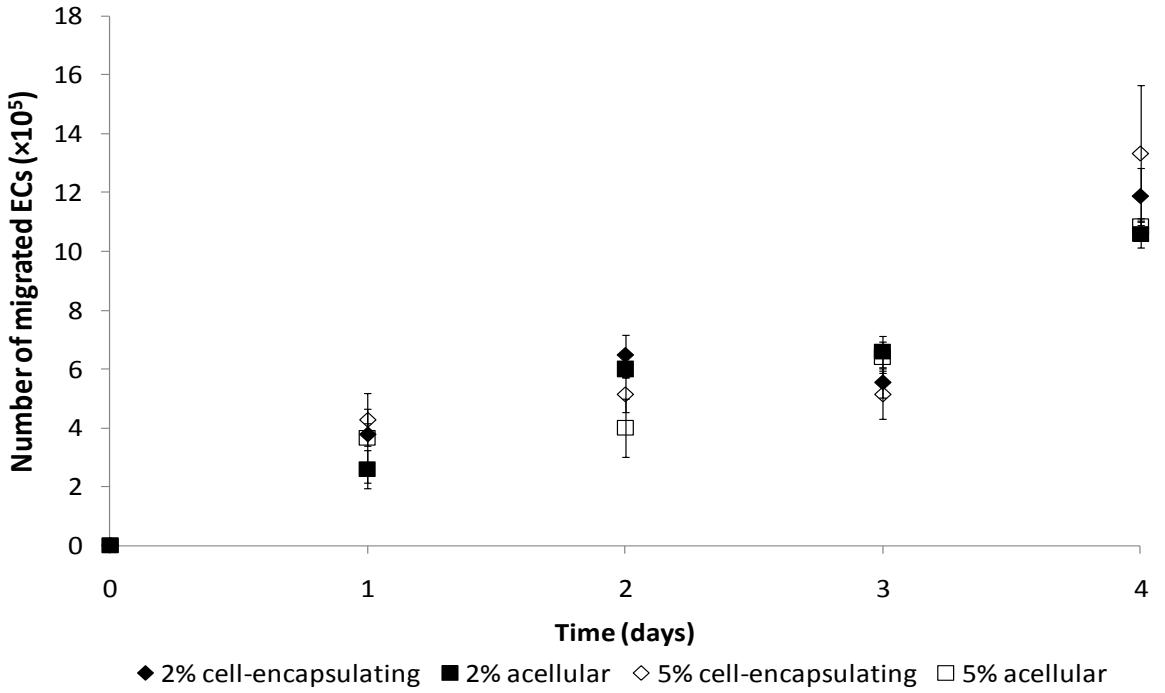



**Figure 8.9: Number of ECs which had migrated through the culture membrane towards acellular or fibroblast-encapsulating samples of 2% and 5% w/v alginate, with time. Mean values ± SD are shown (n=3). The migration of ECs towards all four samples was relatively similar. Significant differences in the number of ECs on the underside of membranes were only seen at day two between 5% acellular and 2% acellular or 2% cell-encapsulating samples.**

### 8.2. Discussion

Angiogenesis is a key requirement for successful tissue engineering of skin. Although fibroblasts are known to secrete angiogenic factors (Chen *et al.* 2004; Tunyogi-Csapo *et al.* 2007), this appears to be the first study evaluating the effect of encapsulation on the secretion of angiogenic factors. RT-PCR analysis indicated that fibroblasts encapsulated in both 5% and 2% w/v alginate should provide sustained release of VEGF, IL-6 and NGF, to support vascularisation of the dermal analogue. Conversely, the transcription of FGF-2 was not seen to be sustained by fibroblasts following encapsulation in the alginate hydrogels (Figure 8.1).

The VEGF ELISA (Figure 8.7), showed that fibroblasts encapsulated in 2% and 5% w/v alginate expressed a constant level of VEGF protein, which was detected in the culture medium surrounding the samples. The level of VEGF expression was, however, approximately four times lower than that seen before encapsulation. The difference in VEGF transcription and expression profiles may be due to the VEGF mRNA not being

stabilized as much as in control samples after transcription in the encapsulated samples. A number of factors are known to be involved in the stabilization of VEGF mRNA, including hypoxia-inducible factor-1alpha (HIF-1α) (White *et al.* 1995) and Bcl-2 (Iervolino *et al.* 2002). Down-regulation of expression of these proteins may be the reason for decreased translation of VEGF following encapsulation, which may occur as a result of the reduced metabolic activity (Adair 2005; Adair *et al.*1990) of the encapsulated fibroblasts (Figures 6.8 and 6.11).

In order to assess whether the combined levels of angiogenic factors secreted by encapsulated fibroblasts were sufficient to induce angiogenesis, the effect of the encapsulated cells on the migration and proliferation of endothelial cells were assessed. It was seen that the fibroblasts encapsulated in both 2% and 5% w/v alginate had no significant effect ($p>0.05$) on the proliferation or migration of the endothelial cells (Figures 8.8 and 8.9), suggesting that the level of angiogenic factors secreted was too low to induce angiogenesis. Further work should therefore seek to increase the level of angiogenic factor expression by encapsulated fibroblasts.

It is, however, possible that the apparent lack of angiogenic response to the encapsulated fibroblasts *in vitro* is not representative of the *in vivo* response (Egginton 2009). VEGF (Breen 2007) and IL-6 (Egginton 2009) are also known to induce leukocyte recruitment which also secrete VEGF (Nogami *et al.* 2007) to induce angiogenesis (Sidky & Auerbach 1975; Sidky & Auerbach 1976). Further work should therefore seek to assess whether the level of VEGF and IL-6 expression are sufficient to induce leukocyte proliferation and migration. In addition, the levels of expression of these proteins may be sufficient to facilitate other aspects of wound healing. For example, VEGF and NGF can promote the regeneration of peripheral nerve as well as contraction and re-epithelisation of wound (Hu *et al.* 2007).

Furthermore, only the angiogenic potential of fibroblasts in the dermal analogue have been assessed. Keratinocytes are also known to secrete angiogenic factors including VEGF (Detmar *et al.* 1995; Detmar 2000). VEGF expression by keratinocytes is seen to occur at increasing levels with increased stratification (Shirakata *et al.* 2003). Proliferating keratinocytes in the basal layer of the epidermis also secrete TGF-alpha (Werner *et al.* 2007) which is known to stimulate endothelial cell migration (Grotendorst *et al.* 1989) and to up-

regulate VEGF expression by keratinocytes (Detmar *et al.* 1995), and thus also promotes enhanced vascularisation. Further work should seek to evaluate the potential of keratinocytes in the tissue engineered epidermal layer to induce angiogenesis.

### 8.3. Conclusions

In this chapter it has been demonstrated that fibroblasts encapsulated in alginate hydrogel can provide sustained release of VEGF, although at a level lower than before encapsulation. Since sustained IL-6 and NGF expression were also recognised following encapsulation in alginate hydrogels, sustained release of these proteins may also be achieved. Although the level of angiogenic factor expression was not seen to be sufficient to induce proliferation and migration of endothelial cells *in vitro*, this may be achieved in vivo due to recruitment of VEGF-secreting leucocytes. In addition, since VEGF and NGF are also known to enhance other aspects of wound healing, such as epithelialisation, collagen deposition and innervation, the results indicate that many aspects of wound healing may be enhanced by fibroblasts encapsulated in alginate hydrogel.

# 9. Re-epithelialisation

In addition to the restoration of the ECM of the dermal matrix, and the vascularisation of the dermal layer, it is essential that the wound site becomes re-epithelialised to prevent penetration of foreign materials and prevent heat and water loss from the body (Gibbs & Ponec 2000; Liu *et al.* 2007). Re-epithelialisation occurs by the proliferation of keratinocyte basal cells and their progressive differentiation to form the suprabasal layers and finally the stratum corneum (SC). The differentiation of the keratinocytes can be evidenced by examining the expression of various cytokeratins (Ks) and other proteins expressed in the specific strata of the epidermis (Fuchs 2007). The cells in the basal layer express K5, K14 and ki67 (Gibbs & Ponec 2000; Nelson & Sun 1983), whilst the spinous layer cells express K1 and K10 (Fuchs 2008). Involucrin (INV) and fillagrin (FGN) are expressed in the upper most region of the suprabasal layers shortly before the cells lose their nuclei and form the SC, and can be used as an indication of SC presence (Fuchs 2008; Gibbs & Ponec 2000). The proliferation and differentiation of keratinocytes is controlled by paracrine interactions with the fibroblasts of the dermis.

The fibroblasts secrete various growth factors which stimulate re-epithelialisation including keratinocyte growth factor (KGF), granular macrophage colony stimulating factor (GM-CSF), pleiotrophin (PTN), transforming growth factor β (TGFβ), interleukin-6, vascular endothelial growth factor (IL-6), vascular endothelial growth factor (VEGF) and stromal cell-derived factor (SDF-1) (Braunstein *et al.* 1994; Finch & Rubin 2004; Florin *et al.* 2005; Grossman *et al.* 1989; Singer & Clark 1999; Werner *et al.* 2007). In Chapter 8 the expression of IL-6 and VEGF by encapsulated fibroblasts was demonstrated, indicating that the alginate encapsulated fibroblasts should support epithelialisation.

In this chapter the potential of the bi-layered tissue engineered skin to support re-epithelialisation of a wound site was further assessed. The ability of the fibroblasts in the dermal analogue to support keratinocyte proliferation and differentiation on the surface to produce a stratified epidermal layer was assessed by semi quantitative RT-PCR analysis of KGF expression, compared with the expression of these factors by fibroblast monolayers. The formation of multi-layered epidermal layer on the surface of the alginate/ fibroblast dermal analogue was assessed and compared with normal skin and keratinocytes grown at the air/liquid interface (A/L) on Thincert polystyrene polymer membranes in the presence of a

fibroblast feeder layer. The differentiation of keratinocytes was assessed by RT-PCR analysis of keratins (Ks) 1, 5, 14 and involucucrin. Histological staining of the epidermal layers with H&E allowed tissue architecture to be assessed and IHC staining for Pan cytokeratin (PanK) was performed to confirm that the stratified layers were formed of keratinocytes, since co-cultures of fibroblasts and keratinocytes were used and primary keratinocytes were isolated from rats, and therefore contamination with other cells was possible.

## 9.1. Results

### 9.1.1. RT-PCR analysis of KGF expression by fibroblasts following encapsulation

The potential of fibroblasts in the dermal analogue to support the growth and differentiation of keratinocytes on the surface was assessed by RT-PCR analysis of KGF expression, compared with GAPDH expression (Figure 9.1). The expression of KGF was assessed at one and three weeks post encapsulation, while still encapsulated, or following release from encapsulation. Control samples were monolayer cultures of fibroblasts which had not been previously encapsulated in alginate hydrogel. Visual examination showed that KGF was expressed by fibroblasts in all five conditions. The relative transcription KGF under the different conditions was determined and is represented in Figure 9.2.

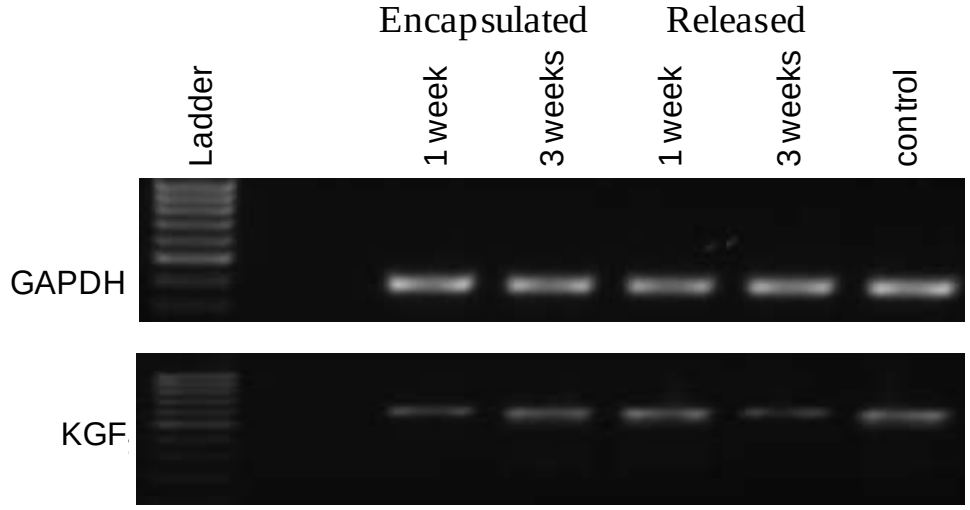



**Figure 9.1: RT-PCR analysis of GAPDH and KGF expression by 3T3 fibroblasts encapsulated in 5% w/v alginate hydrogel for one and three weeks. Expression is shown for encapsulated cells and those which are released and grown as monolayers for 48h, compared with culture as monolayers for 48h before encapsulation (control). KGF was expressed at week one and three while encapsulated and following release from alginate hydrogel encapsulation.**

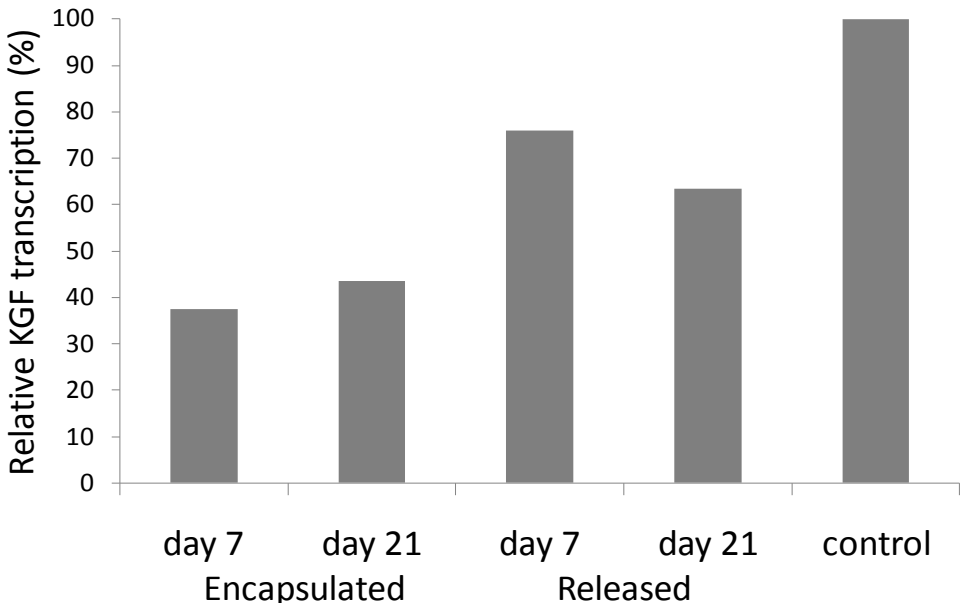



**Figure 9.2: Relative KGF transcription by 3T3 fibroblasts encapsulated and released from 5% w/v alginate hydrogel after one and three weeks compared with monolayer cultures of fibroblasts which had not been encapsulated (control). Cells which were released from the alginate were grown for 48h in monolayer culture before RT-PCR analysis. KGF transcription was maintained following encapsulation in 5% w/v alginate hydrogel, although the level of transcription was reduced by at least 50% during encapsulation and by ~20-35% after release from encapsulation.**

KGF transcription was down regulated by more than 50% during encapsulation when compared with the control (Figure 9.2). Following release from the alginate hydrogel at weeks one and three the transcription of KGF increased considerably when compared with that during encapsulation, but was still ~20-35% lower than in control samples.

### 9.1.2. Formation of the epidermis by keratinocytes

The growth and stratification of keratinocytes on the surface of the dermal analogue was assessed by RT-PCR, histological analysis and IHC.

#### 9.1.2.1. Histology and Immunochemistry

After two weeks of keratinocyte growth at the A/L sections of the TE epidermis formed on the surface of the alginate/ fibroblast dermal analogue were stained with H&E and compared with sections of normal rat skin and TE epidermis formed on the surface of Thincert™ membranes (Figure 9.3). The results indicate that after two weeks of culture at the A/L,

keratinocytes on both the dermal analogue and the Thincert™ membrane had formed multi-layered structures. The TE epidermis formed on the dermal analogue, however displayed no evidence of a SC, where as the tissue engineered epidermis formed on the surface of the membrane appeared to be fully stratified with presence of a SC, and closely resembled the epidermis of normal skin (9.3a). The multi-layered epidermal layers formed on the surface of the dermal analogue and the membrane were confirmed to be formed from keratinocytes rather than fibroblasts or other contaminating cell types by IHC staining for PanK (Figure 9.4). The presence of PanK in the tissue engineered epidermal layers (Figures 9.4b and 9.4c) was indicated by the brown staining of the cells, as was seen in the epidermis of normal skin (Figure 9.4a). The cells in the dermal layer did not stain positive for PanK, since the cells in this layer are mainly fibroblasts and not keratinocytes.

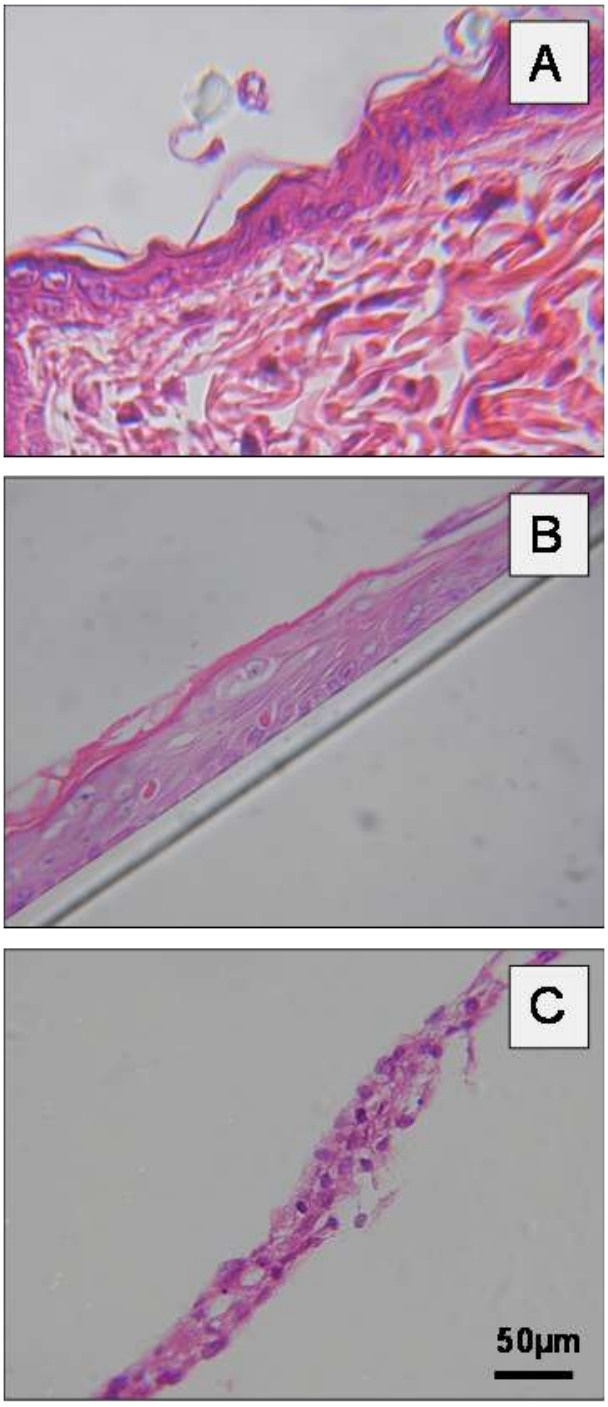



**Figure 9.3: H&E staining of a) normal skin showing epidermal architecture, and TE epidermis grown at the A/L for 14 days on the surface of b) a Thincert™ polystyrene polymer membrane in the presence of a 3T3 feeder layer and c) 5% w/v alginate/ 3T3 dermal analogue. A muli-layered structure was formed on the surface of the dermal analogue, but after 14 days there was no evidence of a SC. A SC was present in the control TE epidermis, which closely resembled the epidermis of normal rat skin.**

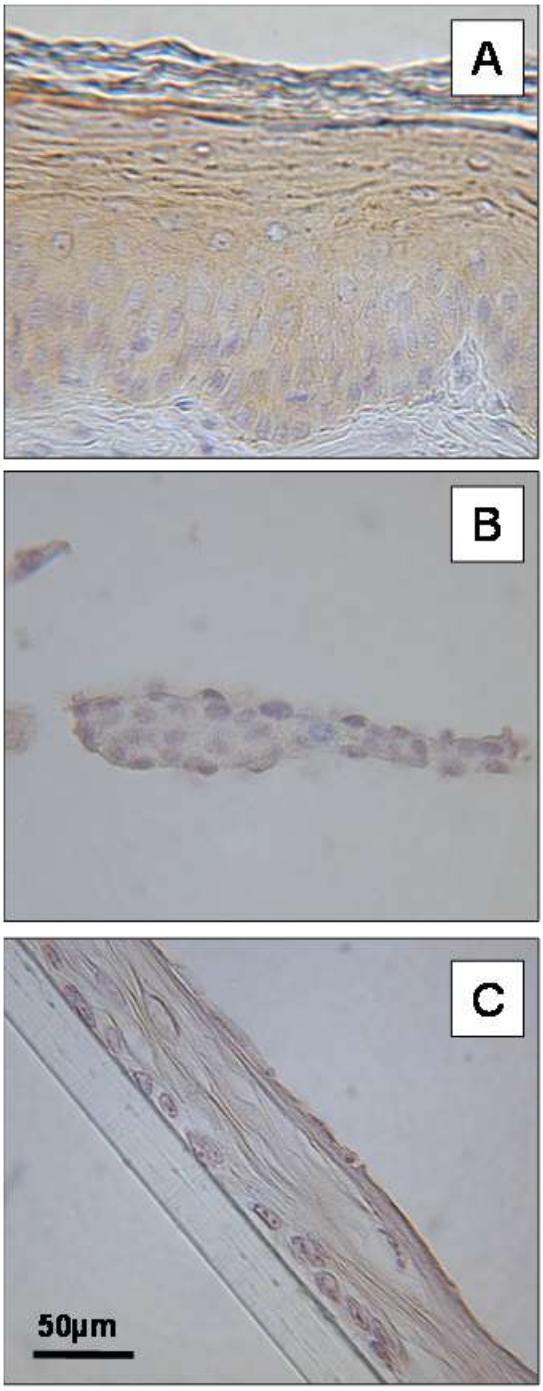



**Figure 9.4: PanK staining of a) normal rat skin and the TE epidermis formed on the surface of the b) dermal analogue and c) Thincert membrane after 14 days culture at the A/L interface. Positive staining for PanK is indicated by brown staining of the cells, as can be seen for the majority of cells in the epidermis of normal skin (a), in which the main cell type is the keratinocyte. No brown staining is seen in the dermis of normal skin (a), where keratinocytes are not present. The positive staining for PanK in (b) and (c) indicated that the cell layers formed are indeed composed of keratinocytes and not other contaminating cell types such as fibroblasts.**

### 9.1.2.2. RT-PCR analyisis of keratinocyte differentiation markers

In order to determine the presence of distinct epidermal sub-layers in the TE epidermal layers, expression of K5, K14, K1 and involucrin by keratinocytes in the TE epidermal layers was assessed by RT-PCR and compared with the expression of GAPDH after four, 7, 11 and 14 days culture at the A/L. Monolayer cultures of fibroblasts were used as a negative control to show the primers were specific to the target proteins which are only expressed by keratinocytes and not by fibroblasts (Figure 9.5). The relative expression of K5, K14, K1 and involucrin were determined by measuring the relative band intensities.

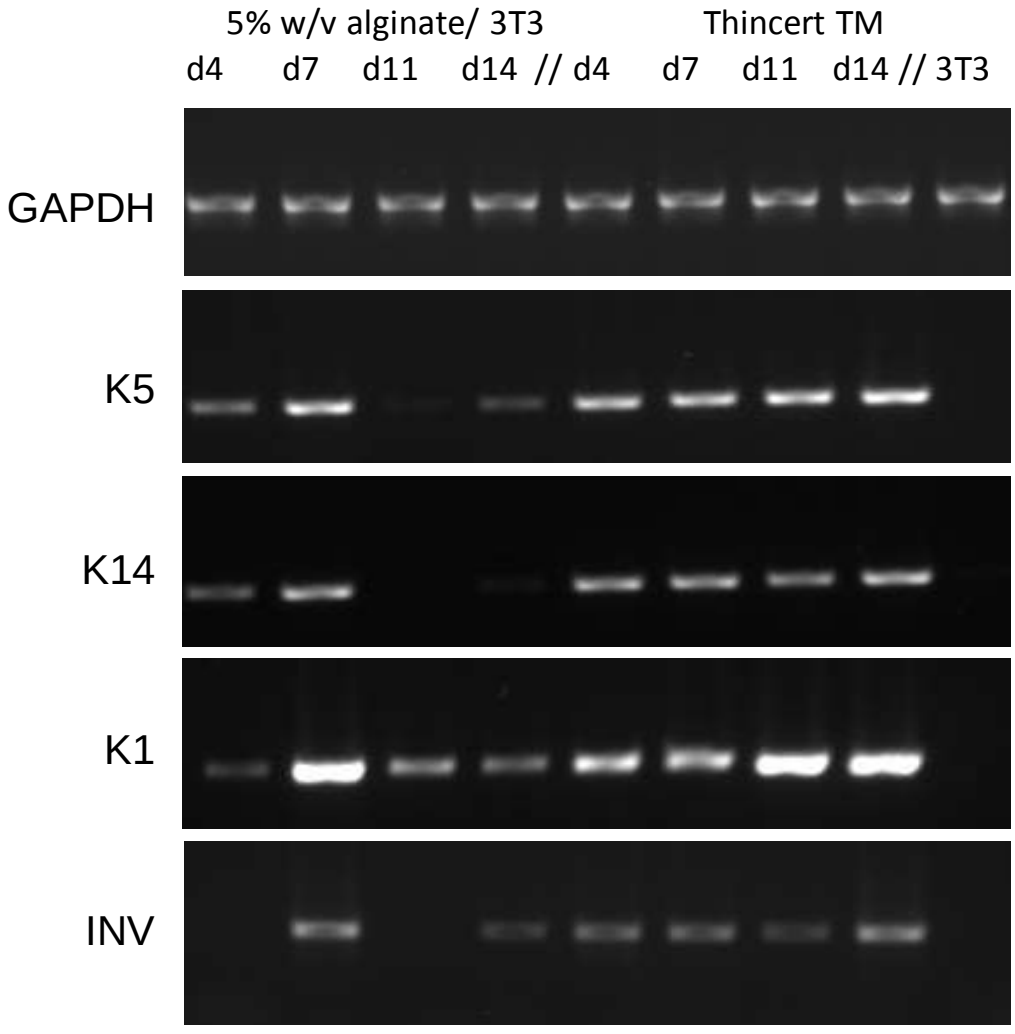



**Figure 9.5: GAPDH, Keratin 1, 5, 14 and Involucrin expression by keratinocytes grown at the A/L for 4, 7, 11 and 14 days. Keratinocytes were either grown on alginate/ 3T3 dermal analogue (with no feeder layer) or Thincert™ polymer membrane in the presence of a feeder layer. No expression of Keratin 1, 5, 14 and Involucrin by 3T3 fibroblasts demonstrated that the primers were specific to these keratinocyte-expressed genes.**

K5 is a marker of the basal layers of the epidermis, and this gene was expressed at all time points in both the epidermal tissue formed on the Thincert™ membrane and on the dermal analogue (Figure 9.6). On the surface of the dermal analogue, the level of K5 expression increased between days four and seven, before dropping between days 7 and 11, then increasing again between days 11 and 14. The expression of K5 was constant between day four and day 7 then doubled between day 7 and day 14. K14 is also a basal cell marker, and as expected, the pattern of K14 expression (Figure 9.7) was very similar to that of K5 (Figure 9. 6).

The presence of suprabasal layers in the samples was identified by detection of K1 expression (Figure 9.8). K1 expression at day four and 7 for the dermal analogue samples was comparable with the transcription patterns seen for K5 and K14. For the membrane-cultured samples, the expression of K1 was strong at all four time points indicating presence of suprabasal layers at all four time points.

Involucrin expression at day four was not evident with the dermal analogue samples but was strong at day 7 indicating the presence of cells which had terminally differentiated (Figure 9.9). The expression of involucrin was then not detected at day 11 but was detected at day 14, although at a level which was only approximately 50% of that at day 7. Involucrin expression for keratinocytes cultured on Thincerts was detected at all four time points, although it increased between day four and 14 illustrating that more cells were terminally differentiating with time, as would be expected as stratification occurs. A decrease in involucrin expression was, however, seen between day 7 and day 11.

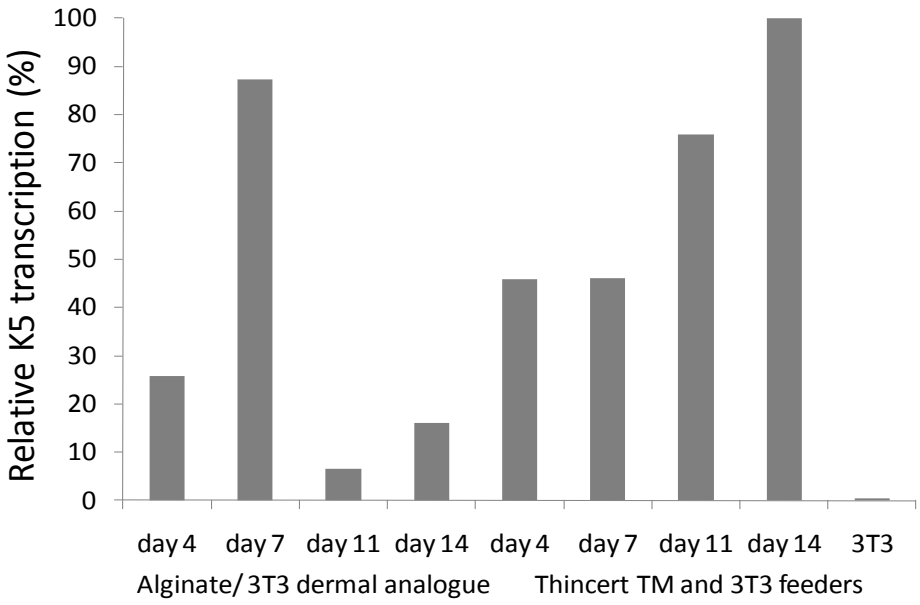



**Figure 9.6: Relative expression of K5 by keratinocytes grown at the A/L for 4, 7, 11 and 14 days. Keratinocytes were either grown on the alginate/ 3T3 dermal analogue (with no feeder layer) or Thincert™ polymer membrane in the presence of a feeder layer. 3T3 fibroblasts were used as a negative control. K5 expression indicated presence of basal layer keratinocytes.**

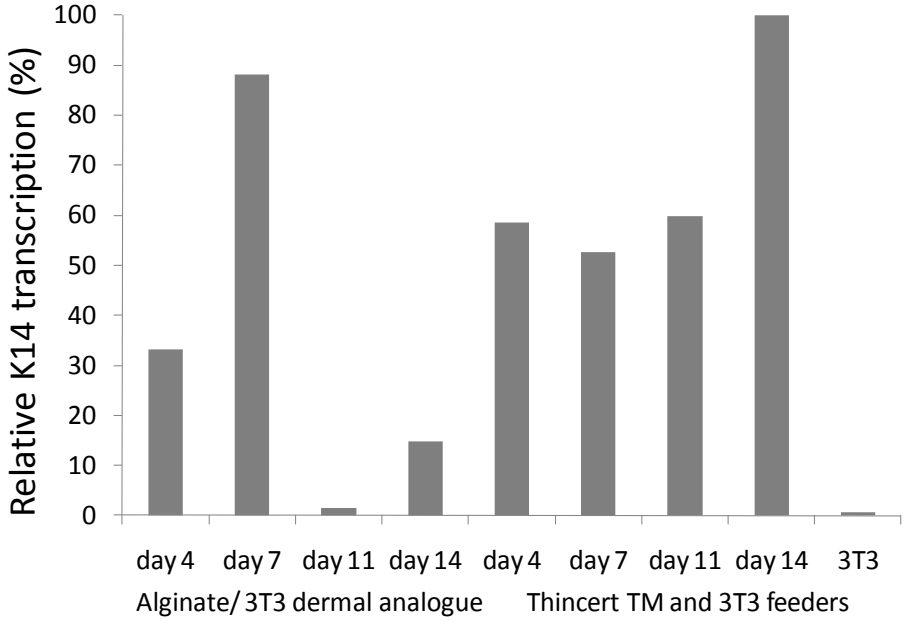



**Figure 9.7: Relative expression of K14 by keratinocytes grown at the A/L for 4, 7, 11 and 14 days. Keratinocytes were either grown on the alginate/ 3T3 dermal analogue (with no feeder layer) or Thincert™ polymer membrane in the presence of a feeder layer. 3T3 fibroblasts were used as a negative control. K14 expression indicated the presence of basal layer keratinocytes.**

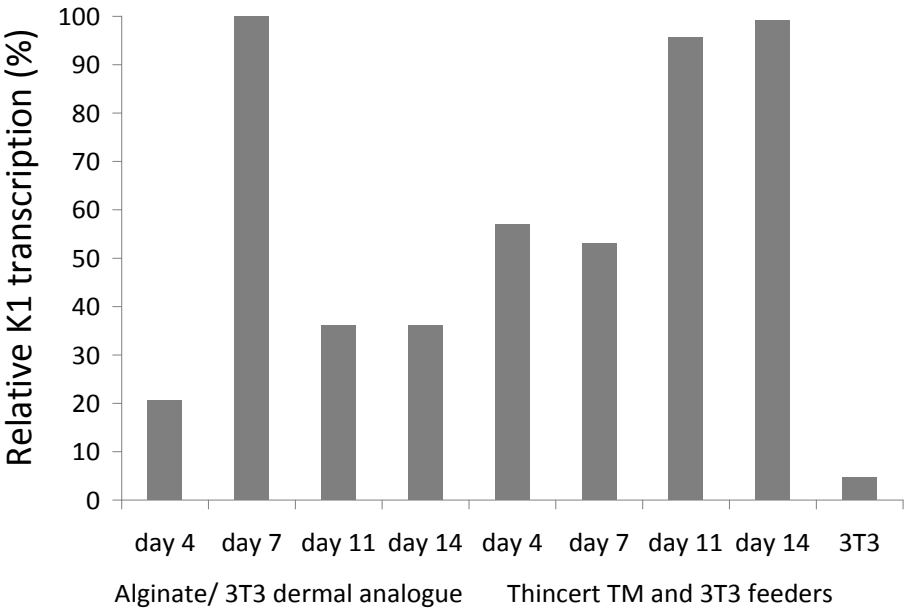



**Figure 9.8: Relative expression of K1 by keratinocytes grown at the A/L for 4, 7, 11 and 14 days. Keratinocytes were either grown on the alginate/ 3T3 dermal analogue (with no feeder layer) or Thincert™ polymer membrane in the presence of a feeder layer. 3T3 fibroblasts were used as a negative control. K1 expression indicated presence of suprabasal layer keratinocytes.**

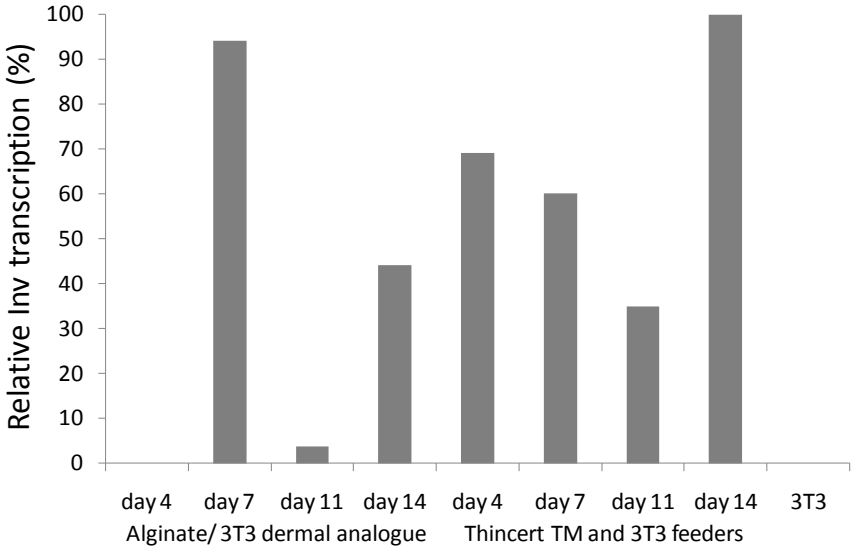



**Figure 9.9: Relative expression of Involucrin by keratinocytes grown at the A/L for 4, 7, 11 and 14 days. Keratinocytes were either grown on the alginate/ 3T3 dermal analogue (with no feeder layer) or Thincert™ polymer membrane in the presence of a feeder layer. 3T3 fibroblasts were used as a negative control. Involucrin is a component of the cornified envelope and expression of involucrin indicated the presence of terminally differentiated keratinocytes of the suprabasal layers, progressing toward the SC.**

## 9.2. Discussion

A key requirement of skin healing is the effective re-epithelialisation of the wound site (Brem *et al.* 2009; Stojadinovic *et al.* 2007). The epidermis forms by the proliferation of basal stem cells to cover the wound surface and the progressive differentiation of these basal cells to form the cells of the suprabasal layer, which later terminally differentiate to form the SC (MacNeil 2007). Effective restoration of the epidermis, however, requires the presence of both keratinocytes and fibroblasts (Fuchs 1990; Fuchs 2008). For this reason, when tissue engineered epidermis is produced *in vitro*, mitomycin-treated or lethally irradiated post-mitotic cultures of fibroblasts are often used as feeder layers (Maas-Szabowski *et al.* 2000;Werner *et al.* 2007; Witte & Kao 2005) to provide growth factors and cytokines to support epithelial regeneration. For this reason a mitomycin-treated fibroblast feeder layer was used to support epithelial development in control samples of keratinocytes grown on Thincert™ polymer membranes.

For the production of the TE epidermis on the surface of the dermal analogue, the fibroblast feeder layer was eliminated since fibroblasts were present in the dermal analogue and normal fibroblasts encapsulated in fibrin have previously been shown to act as an effective feeder layer for the production of tissue engineered epidermis (Panacchia *et al.* 2010; Rheinwald & Green 1975b). Therefore, it was proposed that the alginate encapsulated fibroblasts should also act as an effective feeder layer, especially since the alginate encapsulated fibroblasts in the dermal analogue have been shown to be mitotically inhibited (Figures 6.3 and 6.7), and therefore should not overgrow the keratinocytes in the co-culture, and remain viable for at least 150 days encapsulation (Figure 6.1). The potential of the fibroblasts in the dermal analogue to act as feeder layers was further demonstrated by their sustained transcription of IL-6, VEGF (Figures 8.1, 8.3 and 8.6) and KGF (Figures 9.1 and 9.2) following encapsulation in 5% w/v alginate hydrogel, relative to control samples of monolayer cultured fibroblasts. Continued transcription of these factors was seen following encapsulation in the alginate hydrogel, as has been shown for fibroblasts cultured in collagen-based dermal analogues (Panacchia *et al.* 2010). This indicated that the dermal analogue should support keratinocyte growth and differentiation to restore the epidermis (Froget *et al.* 2003; Sun *et al.* 2009). Alginate hydrogel may, therefore, be a suitable alternative scaffold material to the collagen-based scaffolds commonly used in dermal TE (Finch & Rubin 2004; Grossman *et al.* 1989; Werner *et al.* 2007).

The keratinocytes which were cultivated on the surface of the dermal analogue were seen to form a multi-layered epidermal layer after 14 days A/L culture (Figures 9.3 and 9.4). The epidermal layer, however, lacked a SC and was seen loosely packed in comparison with the TE epidermis formed after 14 days culture at the A/L on the surface of the Thincert™ membrane and the epidermis of normal rat skin. In addition, the epidermal layer formed of the TE bi-layer was not seen to cover the entire surface of the dermal analogue and RT-PCR analysis of these differentiation markers indicated that keratinocytes grown at the A/L on the surface of the dermal analogue proliferated and differentiated more extensively within the first 7 days than keratinocytes grown on the Thincert™ membrane. In the following 7 days culture, the keratinocytes grown on the dermal analogues showed decreased expression of all markers, compared with the control samples (Figures 9.5-9.9).

Keratinocyte adhesion to the growth substrate regulates differentiation of the keratinocytes to form a stratified epidermis (Jensen & Wheelock 1996). The altered expression pattern of keratinocyte differentiation markers, and epidermal architecture, in comparison with the membrane-cultures TE epidermis is, therefore, likely due to the limited cell adhesiveness of the alginate hydrogel (Braunstein *et al.* 2004; Florin *et al.* 2005; Grossman et *al.* 1989; Singer & Clark 1999; Werner *et al.* 2007).

### 9.3. Conclusion

The potential of fibroblasts in the dermal analogues to act as a feeder layer to support epidermal regeneration has been demonstrated by the sustained transcription of VEGF, IL-6 and KGF following encapsulation in 5% w/v alginate hydrogel. Keratinocytes cultured on the surface of the dermal analogue produced multi-layered epidermal layers. The differentiation pattern of the keratinocytes and the morphology of the TE epidermis of the bi-layer construct was altered compared with TE epidermis grown on Thincert™ membranes in the presence of mitomycin-treated feeder layers. This may result from the limited adhesion of keratinocytes to the alginate surface.

# 10. Conclusions and future work

## 10.1. Conclusions

In this thesis, a TE skin bi-layer consisting of fibroblasts encapsulated in calcium-alginate hydrogel and keratinocytes seeded on the surface has been developed and characterised. The effectiveness of the TE skin bi-layer to promote dermal regeneration, re-epithelialisation and vascularisation has been assessed *in vitro*. The main conclusions of each results chapter are described below.

### 10.1.1. Effect of encapsulation on fibroblast viability, growth and catabolic activity

Fibroblasts encapsulated in 5% and 2% w/v alginate hydrogels were shown to remain viable for at least 150 days encapsulation. This is essential to ensuring that these fibroblasts can secrete factors to support keratinocyte growth and differentiation to promote re-epithelialisation, secrete ECM to provide dermal repair and secrete factors to support endothelial recruitment to facilitate vascularisation of the tissue.

Alginate encapsulation was also shown to cause mitotic inhibition. This mitotic inhibition was seen to be reversed by degradation of the alginate hydrogel scaffolds to release the encapsulated fibroblasts, confirming that viability of encapsulated fibroblasts was maintained. The mitotic inhibition indicated that the encapsulated fibroblasts should not over-grow the keratinocytes in the co-culture, without the need for gamma-irradiation or Mitomycin C treatment of the fibroblasts.

The reversible mitotic inhibition of fibroblasts encapsulated in alginate hydrogels was accompanied by reversible catabolic inhibition of the cells. This catabolic inhibition was likely the reason that the viability of the cells was maintained for at least 150 days encapsulation in the avascular scaffold. The reversible nature of the metabolic inhibition again confirmed that the viability of the cells was maintained following encapsulation. It also demonstrated that as the scaffold degraded *in vivo* the metabolic demand of the tissue would increase and, therefore, it is essential that the scaffold should become vascularised.

### 10.1.2. Replacement of alginate hydrogel scaffold with normal ECM

During encapsulation, histological evaluation displayed no evidence of ECM accumulation, but following degradation of the alginate hydrogels, encapsulated fibroblasts were seen to

secrete collagen. The alginate hydrogel scaffold should, therefore, be replaced with normal ECM to facilitate dermal repair. The degradation of acellular and fibroblast-encapsulating 2% and 5% w/v alginate hydrogels were assessed by measuring release of cross-linking calcium cations by inductively coupled plasma mass spectroscopy (ICP-MS), and changes in rheological properties, morphology and mass. The results indicated that the alginate hydrogels degraded slowly over time and retained gel-like morphology and mechanical properties for at least 28 days culture. There was a steep decline in mechanical properties of all alginate hydrogels within the first 7 days, followed by a slower decline throughout the following 21 days. The decline in mechanical properties during the first 7 days was increased by the inclusion of cells in the scaffolds, but the differences in mechanical properties between acellular and cell-encapsulating samples could not be attributed to differences in the calcium release profiles between cell encapsulating and acellular samples. Overall, the results of degradation analysis indicated that 5% w/v alginate hydrogel degraded more slowly than 2% w/v alginate hydrogel, and retained higher mechanical properties over the culture period. 5% w/v alginate hydrogel should, therefore, better support the regeneration of the dermal and epidermal layers and was, therefore, chosen as the scaffold material for construction of the bi-layer skin replacement.

### 10.1.3. Angiogenesis

Scaffold vascularisation is essential to maintaining the viability and normal function of transplanted cells, to support tissue development. Graft vascularisation is known to be achieved by angiogenesis, which is promoted by angiogenic factors such as FGF-2, VEGF, IL-6 and NGF. RT-PCR analysis demonstrated that sustained transcription of VEGF, IL-6 and NGF were maintained by fibroblasts following encapsulation in both 2% and 5% w/v alginate hydrogels. The transcription of FGF-2, however, was not maintained. Since VEGF is the main factor responsible for promotion of angiogenesis, the level of VEGF expression by encapsulated fibroblasts was also assessed by ELISA. The level of VEGF protein expression by fibroblasts encapsulated in both 2% and 5% w/v alginate hydrogels was shown to be comparable, but approximately four times lower than the level of expression in monolayer fibroblast cultures. As expected, the level of expression of angiogenic factors was not high enough to cause enhanced endothelial cell recruitment or migration toward the alginate hydrogel scaffolds.

### 10.1.4. Re-epithelialisation

Fibroblasts encapsulated in 5% w/v alginate hydrogel were shown by RT-PCR to maintain transcription of KGF, which in addition to IL-6 and NGF, which were shown to be transcribed in Chapter 8, is known to support the proliferation and differentiation of keratinocytes to achieve epidermal replacement. The formation of a multi-layered epidermal layer by keratinocytes seeded on the surface of the alginate hydrogel was compared with that of keratinocytes seeded onto a Thincert™ membrane in the presence of a Mitomycin C-treated feeder layer. Histological analysis and PanK staining at day 14 after A/L culture indicated that the keratinocytes formed multi-layered epidermal layers on the surface of the alginate hydrogel. The layers, however, were not continuous, and appeared to lack a stratum corneum. In addition, RT-PCR of differentiation markers indicated that keratinocytes grown on the surface of the alginate had an altered differentiation pattern compared with keratinocytes grown on Thincert™ membranes. This was likely due to the limited adhesion of keratinocytes to the surface of the alginate hydrogel, since adhesion is a key regulator of epidermal development.

## 10.2. Future work

Although the potential of the bi-layer to enhance wound healing has been illustrated, it is clear that further assessment of this potential should be assessed. Further work should seek to quantify the level of KGF, NGF and IL-6 protein expression by alginate encapsulated fibroblasts, to assess whether it corresponds with the transcription of these genes. The expression of GM-CSF, PTN, TGFβ, and SDF-1, (Mansbridge 2008) should also be quantified in order that the potential of alginate-encapsulated fibroblasts to promote re-epithelialisation can be better understood. The effect of the TE skin when seeded with autologous keratinocytes and fibroblasts, on wound healing *in vivo* should be studied. This should include analysis of the inflammatory response, scaffold degradation, ECM accumulation in the dermal layer, vascularisation, cell viability, cell migration, epidermal development, wound contraction, scar formation and innervation of the wound site (Babensee *et al.* 2003).

The results also seem to indicate that further improvements should be made to the TE skin to enhance wound healing. In particular, efforts should be made to enhance keratinocyte adhesion to the surface of the dermal analogue to improve epidermal regeneration, as well as

to increase ECM accumulation in the alginate hydrogel by fibroblasts and enhance angiogenic gene expression by the encapsulated fibroblasts.

### 10.2.1. Increasing keratinocyte adhesion to the dermal analogue

Promoting better adhesion of keratinocytes to the dermal analogue surface would possibly improve the potential for the formation of a full-thickness epidermis, since keratinocyte adhesion to the growth substrate regulates differentiation of the keratinocytes to form a stratified epidermis (Jensen & Wheelock 1996). It has been shown that a higher G:M ratio alginate promotes increased cell adhesion (Wang *et al.* 2003) but cell adhesion and growth even on high G:M alginate hydrogel cross-linked with calcium without any modifications seems to be limited. It is possible that a fibrin-rich blood clot would form around the scaffold *in vivo* to promote better adhesion (Wilshaw *et al.* 2009) of keratinocytes to the surface. Alginate can also be coated with Poly-L-lysine (PLL) (Lu *et al.* 2009; Wittmer *et al.* 2008) or chitosan (Majima *et al.* 2005) to promote cell adhesion of keratinocytes to the surface of the dermal analogue by immersion of alginate hydrogel in a solution of PLL or chitosan, since both PLL and chitosan are positively charged (Semenov *et al.* 2009) and alginate is negatively charged (Rinaudo 2008).

Increased keratinocyte adhesion may also be achieved by increased adhesion throughout the scaffold, which may be achieved in a number of ways, including cross-linking alginate hydrogel with iron (hida-Sano *et al.* 2009) rather than calcium or using a blended biolpolymer gel of alginate and chitosan (Li *et al.* 2005; Majima *et al.* 2005), or covalently modifying the alginate with RGD (Alsberg *et al.* 2001; Augst *et al.* 2006; Kong *et al.* 2003; Rowley *et al.* 1999).

### 10.2.2. Mechanical conditioning

All cells are able to convert inputs from mechanical forces into chemical signals that alter cell migration, proliferation, orientation, metabolism and survival (Cukierman *et al.* 2001). Integrin receptors have been shown to transmit mechanical signals from the ECM to the cell cytoskeleton at structures known as focal adhesions (Nishimura *et al.* 2009). Increasing adhesiveness throughout the alginate hydrogel scaffold would increase the adhesion of fibroblasts and ECs in the dermal layer. This would allow the fibroblasts and ECs to show a greater response to mechanical stimulation (Discher *et al.* 2005), in order that dermal matrix accumulation and angiogenesis may be enhanced. Mechanical conditioning of the TE skin

may therefore increase neo-tissue formation. Mechanical stimulation can be applied in compression (Villanueva *et al.* 2009; Waldman *et al.* 2007), shear (Cassell *et al.* 2002; Steward *et al.* 2010), tensile straining (Deng *et al.* 2009), but ultrasound may be particularly beneficial.

Ultrasound is known to be effective in stimulating fracture (ter Haar 2007), muscle (Piedade *et al.* 2008), skin wound (Altomare *et al.* 2009) and cartilage repair (Speed 2001) and therefore, has been investigated as a method for mechanically conditioning engineered tissues (Bilodeau & Mantovani 2006). Pulsed low intensity ultrasound has been shown to increase matrix synthesis by bovine invertebral disc cells encapsulated in alginate beads (Miyamoto *et al.* 2005), and has been proposed as an effective method for stimulating dentine repair (Scheven *et al.* 2007; Scheven *et al.* 2009b). Ultrasound treatment has also been shown to up-regulate VEGF expression in monolayer cultures of cells (Kobayashi *et al.* 2009; Scheven *et al.* 2009a), and has been shown to enhance the formation of new blood vessels in full-thickness excised lesions (Emsen 2007) and therefore, may enhance the production of angiogenic factors by encapsulated fibroblasts, to expedite vascularisation of the construct. Ultrasound treatment may therefore prove an effective method for improving epithelialisation, matrix accumulation and vascularisation of the tissue engineered skin bi-layer.

### 10.2.3. Chemical stimulation of collagen production

Collagen accumulation can also be enhanced by addition of interferon-gamma, ascorbic acid (Chung *et al.* 1997), TGF-β (Chan *et al.* 2005) and proline (Barbul 2008), which are promoters of the collagen synthesis pathway. The effectiveness of addition of these molecules to culture media to enhance dermal and epidermal repair should be investigated, both in the presence and absence of mechanical stimulation.

### 10.2.4. Angiogenesis

Further work should also seek to analyse whether down-regulation of VEGF protein expression resulted from down-regulation of HIF-1 and Bcl-2 expression. Approaches to enhancing angiogenic gene expression, in addition to mechanical stimulation, should also be investigated. For example, since copper is known to enhance angiogenesis (Barralet *et al.* 2009; Gerard *et al.* 2010) by promoting the transcription of angiogenic factors such as VEGF (Xie & Kang 2009) and IL-8 (Bar-Or *et al.* 2003), and promoting the expression and

stabilizing HIF-1 (Martin *et al.* 2005), enhanced angiogenic gene expression by alginate encapsulated fibroblasts may be achieved by cross-linking some of the alginate with copper rather than calcium. In order to better understand the angiogenic potential of the bi-layer skin graft, the effect of keratinocytes on angiogenesis should be investigated, including the expression of VEGF, and the effect of the keratinocytes on endothelial recruitment.

### 10.3. Summary

This thesis has investigated the use of alginate hydrogel as a scaffold material for the co-culture of fibroblasts and keratinocytes to produce TE skin. The potential of the construct to enhance dermal and epidermal regeneration at wound sites has been shown, and indicates that alginate hydrogel may prove to be an effective scaffold material for the tissue engineering of skin. Further assessment and optimisation of the tissue engineering technique should now be sought in order that clinical application may be achieved.

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## 12. Appendix

### Journal Publications

**Hunt NC**, Smith AM, Gbureck U, Shelton RM and Grover LM. Encapsulation of fibroblasts causes accelerated alginate hydrogel degradation. Acta Biomaterialia. *In press*, 2010.

**Hunt NC**, Grover LM. Cell encapsulation using biopolymer gels for medical applications. Biotechnology Letters. *In press*, 2010.

**Hunt NC**, Shelton RM, Grover, LM. Reversible mitotic and metabolic inhibition following the encapsulation of fibroblasts in alginate hydrogels. Biomaterials 30: 6435–6443, 2009.

**Hunt NC**, Shelton RM, Grover LM. An Alginate Hydrogel Matrix for the Localised Delivery of a Fibroblast/ Keratinocyte Co-Culture. Biotechnology Journal. 4 (5): 730-737, 2009.

### Conference Papers

Muhammad HB, **Hunt NC**, Shelton RM, Grover LM, Ward M. Oddo CM, Recchiuto CT, Beccai L. Incorporation of novel MEMS tactile sensors into tissue engineered skin. The 4th International Conference on Bioinformatics and Biomedical Engineering. Chengdu, China, June 18th - 20th, 2010.

**Hunt NC**, Shelton RM, Cooper PR, Grover LM. An Alginate Based Construct for Tissue Engineering Skin. 8$^{th}$ World Congess of Chemical Engineering. Montreal, Canada, 23$^{rd}$-29$^{th}$ August, 2009.

Mehrban N, **Hunt NC**, Smith AM, Grover LM. A Comparative Study of Iota Carageenan, Kappa Carrageenan and Alginate Hydrogels as Tissue Engineering Scaffolds. 15th Gums and Stabilisers for the Food Industry. Wrexham, UK, 22nd - 26th June 2009.

# Incorporation of novel MEMS tactile sensors into tissue engineered skin

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**Abstract**

**Tactile sensation is important for the performance of daily tasks and prevention of injury. Full thickness wounds of greater than 4cm diameter will not spontaneously heal. Tissue engineering can be used to replace the lost skin, but this skin will often fail to be reinnervated. This results in loss of tactile sensations. Here we have developed a novel, silicon based MicroElectroMechanical Systems (MEMS) sensor array that is capable of detecting tactile experiences of the tissue engineered skin. Tissue engineered skin with tactile experiences can also be useful in the replacement of animal testing in the pharmaceutical industry.**

**Key words: tissue engineering, skin, MEMS tactile sensors, regenerative medicine, pharmaceutical testing.**

## I. INTRODUCTION

Biological tactile sensors of the fingertip play an important role in exploration of surfaces and tactile sensation is important for performance of daily tasks and prevention of injury. The body has a limited capacity to regenerate nerves [1] and although advances have been made in the regeneration of neurons *in vitro* in recent years [2-4], at present fully functional tissue engineered neurons and are not available. Researchers have explored neural interface devices leading to the possibility of re-creating sensory experiences by linking artificial sensing devices with the nervous system [5]. Mechanical tactile sensors have been developed that emulate the performance of mechanoreceptors, i.e. biological touch sensors and there is a general trend towards bio-inspired designs of sensors [6-8].

MEMS technology allows the realization of an array of such sensors that can be incorporated within a small area allowing high spatial resolution and a robust design which is highly desirable in robotics, medical and industrial fields. For packaging such sensors, the general trend has been towards materials mimicking the mechanical properties of skin. To date, materials such as silicone elastomers have been used for packaging sensors [9, 10]. Incorporation of sensors into tissue engineered skin has not been previously explored but is important both within regenerative medicine, in the context of implantable sensors and biohybrid systems [11, 12]. It is also relevant in the pharmaceutical industry for applications where it is important to mimic the contact conditions generated when human skin comes into contact with textured stimuli for characterisation of surfaces. This paper explores the integration of designed silicon based MEMS sensors with tissue engineered skin.

## II. METHODS AND MATERIALS

### A. MEMS Tactile Sensors

A 4 x 1 linear sensor array was developed to measure the distribution of contact forces produced when the tactile device comes into contact with a surface. The main sensing unit consists of a clamped highly doped silicon membrane which, on application of pressure deflects towards a silicon substrate, thereby changing the electrical capacitance between the two plates (Figure 1). The subsequent change in capacitance is measured using a detection circuit. The capacitance as a function of applied force C(F) can be expressed as:

$$C(F) = \int_{x=0}^{a} \int_{y=0}^{b} \frac{\varepsilon}{(d_0 - w(x,y))} dydx \qquad (1)$$

where $w(x,y)$ is the membrane deflection as a function of $x$ and $y$ coordinates of the sensing membrane, $a$ and $b$ are the dimensions of the sensing area, $d_0$ is the initial gap between the two silicon plates and $\varepsilon$ is the permittivity of air.

Sensors were fabricated using standard MEMs fabrication techniques and simple process steps involving photolithography, Deep Reactive Ion Etching (DRIE), Hydrofluoric acid release etching and thermal evaporation of gold. For connection to an electrical measurement system, the fabricated sensors (figure 2) were mounted onto standard DIP chip carriers (figure 3) and wire bonded (using 25 µm aluminium wires). The wires were protected with a coating of epoxy resin. The readout electronics for the tactile sensor array was implemented on a Printed Circuit Board (PCB) and included high resolution capacitance-to-digital converters (AD7747, Analog Devices) having a nominal resolution down to 20 aF. This allowed highly sensitive responses to be measured.

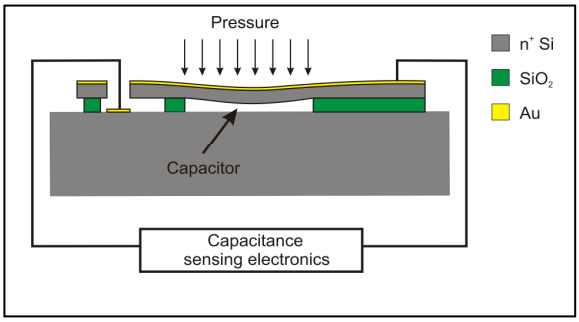


Figure 1. Cross sectional schematic of a single capacitive sensor. On application of pressure, the sensor diaphragm deforms resulting in a change in electrical capacitance between the diaphragm and substrate layer which can be measured using capacitance sensing electronics

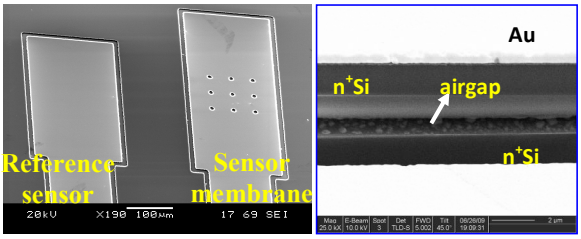


Figure 2. Left – Scanning Electron Micrograph (SEM) image of a single sensor (membrane dimensions 500 µm x 200 µm), Right – cross section of device showing two highly doped Silicon layers separated by an airgap.

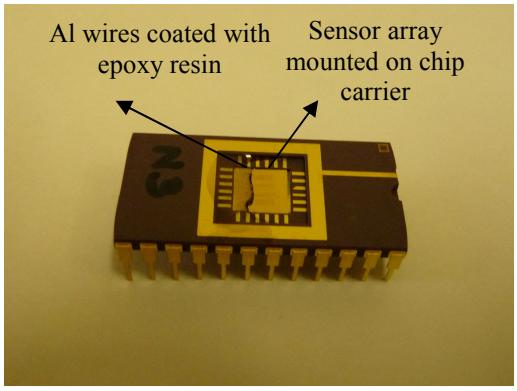


Figure 3. Bare sensor array chip mounted onto standard DIP package. Wires are coated with epoxy to prevent their dislocation during sensor operation.

## B. *Production of tissue engineered (TE) skin*

Keratinocytes were obtained from a neonatal rat sacrificed by cervical dislocation. The keratinocytes were isolated using 0.25% trypsin–0.02% EDTA and mechanical dissociation and cultured in 3:1 DMEM:Ham's F12 supplemented with 50mM hydrocortisone, 10 µg/ml insulin, 25 ng/ml EGF, 10% v/v FBS (PAA), 1% v/v P/S, 2.25% v/v HEPES, 2% v/v L-glutamine as monolayers, with the support of a fibroblast feeder layer prepared by treatment with 4mM Mitomycin C for 2h at 37ºC, until a sufficient number of keratinocytes were obtained, as determined by counting using a haemocytometer. The keratinocytes were seeded at a density of $0.2\times10^6$ cells/cm$^2$ onto Thincert TM membranes and grown in the presence of a Mitomycin-treated feeder layer. The keratinocytes were cultured for a further 7 days on the alginate scaffold before being raised to the air-liquid (A/L) interface for 14 days to allow for stratification of the keratinocytes. All keratinocyte cultures were maintained at 37ºC with 5% $CO_2$ and 100% relative humidity.

Thincert TM polymer membranes were seeded with primary neonatal keratinocytes and after 7 days the keratinocyte culture was raised to the A/L interface for 14 days to promote keratinocyte differentiation in order to produce a full thickness epidermis.

## C. *Characterisation of skin and TE skin by histology*

An adult rat was humanely sacrificed by cervical dislocation before removal of the hairy skin. Tissue engineered epidermis and normal hairy skin samples were fixed in formalin buffer for 24h before progressive dehydration in cassettes (Thermo Shandon, Citadel 1000, Cheshire, UK). The dehydrated samples were then embedded in paraffin wax (Sakura, Tissue Tek, TEC) and stored at room temperature. Samples were then cooled to 5°C and 5µm sections were taken using a microtome (Leica RM 2035), which were attached to glass slides and dried at 60°C in an oven for 1h before removal of the wax in xylene and staining with Haematoxylin and Eosin (H&E) (Surgipath Europe Ltd, Peterborough, UK) to demonstrate tissue architecture. The haematoxylin stained cell nuclei blue/ black whilst the eosin stained the cytoplasm and all extracellular matrix components shades of pink. Coverslips were then applied to the slides using XAM (BDH Laboratory Supplies, Poole, UK) and images of the slides obtained with a light microscope (Carl Zeiss Ltd, Axiolab, Herefordshire, UK) and captured (Canon Powershot GS, Surrey, UK). The images were then corrected using Image J software (NIH, Maryland, USA) to remove any uneven illumination from the image.

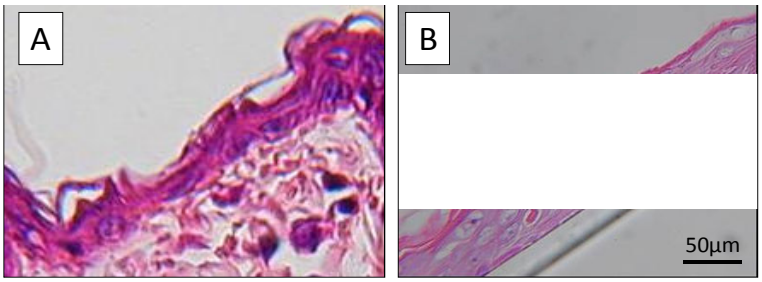


Figure 4. H&E staining of a) normal hairy skin and b) tissue engineered epidermis on Thincert TM polymer membrane. The nuclei of the cells stain blue due to the haematoxylin and cytoplasm and extracellular matrix stain shades of pink due to the eosin. The staining shows that the tissue engineered epidermis is similar in architecture to the epidermis of normal skin with stratification and a stratum corneum.

# III. RESULTS AND DISCUSSION

The fabricated tactile sensors were characterised using load-unload indentation experiments. Controlled normal loads were applied on the surface of the sensor using a loading station with precise position control of a spherical probe. The resulting change in capacitance was simultaneously recorded. Bare sensors were found to be able to resolve forces in the sub mN range as seen in Figure 5.

In robotics sensors are often packaged using materials such as silicone elastomers which are used to simulate skin. To explore performance of the fabricated MEMS sensors with

such packaging, they were they were coated with a layer of Dragon Skin™ Q (Smooth-On, PA, USA). Indentation testing of the sensors coated with this material (Figure 6) indicated that the packaging material reduced the sensitivity of sensors to loading stimulus.

A tissue engineered skin has been produced which was shown by histological staining to be structurally similar in composition to the epidermis of normal skin (Figure 4). The H&E staining illustrated that the keratinocytes had fully stratified to form a 3-5 cell thick epidermis with a stratum corneum. The mechanical properties of skin, tissue engineered skin and Dragon Skin™ Q were compared tribologically and by indentation testing and atomic force microscopy. The mechanical properties of the tissue engineered skin are, in contrast with Dragon Skin™ Q, mechanically similar to normal skin (results not shown). It is therefore thought that coating the sensors with tissue engineered skin rather than Dragon Skin™ Q should allow for the detection and discrimination of tactile stimulation by the MEMS sensors to be more similar to those which would be experienced when the epidermis of normal skin is applied to the surface of the sensors.

Currently work is underway adhering tissue engineered skin and normal skin epidermis directly to the surface of the sensors and carrying out further indentation and characterisation experiments.

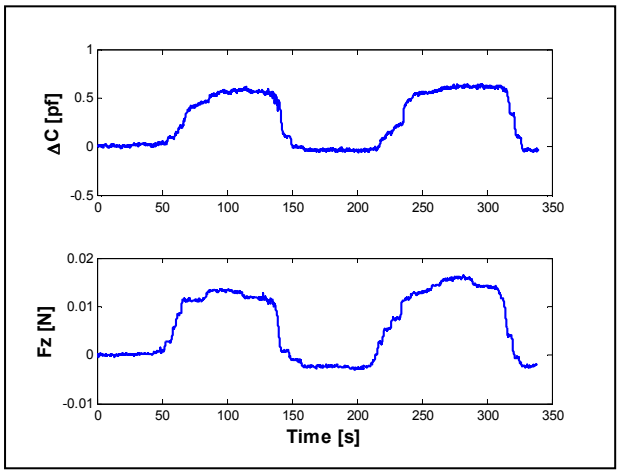


Figure 5. Response of sensors to load - steady state - unload cycles of indentation for bare sensor. Upper trace shows the capacitance change measured during two load-steady state-unload indentation cycles. Bottom trace shows data from commercial load cell ((ATI NANO 17 F/T, Apex, NC, USA). The sensor response is coherent with applied loading conditions. For an applied load of 10 mN there is an approximate capacitance change of 500 fF

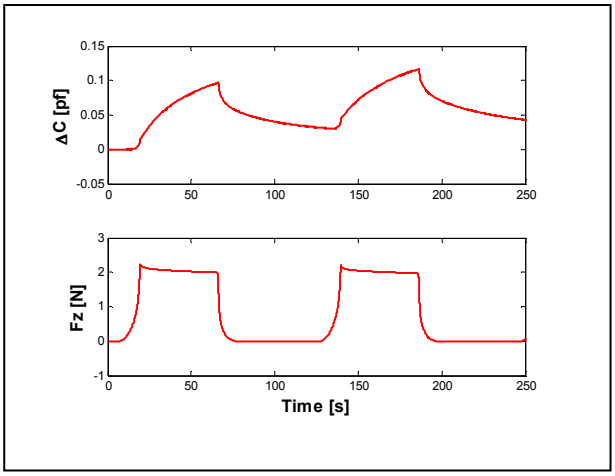


Figure 6. Response of sensors to load- steady state- unload cycle of indentation for sensor packaged with Dragon Skin™ Q . Overall decreased sensitivity and drift of response can be observed during a steady state indentation

## IV. CONCLUSIONS

In this paper, the development and characterization of a silicon based MEMS sensor has been shown, which is capable of detecting forces in the sub mN range. Tissue engineered epidermis has also been developed that can be applied to the surface of these sensors. Tactile stimulation experiments are currently being conducted on MEMS sensors incorporated into tissue engineered epidermis, and the results of these experiments will follow shortly.

## V. ACKNOWLEDGEMENTS

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# AN ALGINATE/ FIBROBLAST/ KERATINOCYTE CONSTRUCT FOR TISSUE ENGINEERING SKIN

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Abstract: Alginate has been used as a biomaterial for around 20 years. It has been used to encapsulate mammalian cells for immunoisolation and tissue engineering applications. In the present study the effectiveness of alginate hydrogel as a delivery vehicle and scaffold for wound healing has been evaluated. Encapsulation of fibroblasts within the alginate hydrogel was shown to cause mitotic inhibition, allowing for keratinocytes seeded on the gel surface to form a stratified epidermal layer that could assist in wound closure. The alginate was shown to degrade over time and in doing so released the encapsulated fibroblasts. The fibroblasts were shown to resume normal growth upon release from the alginate hydrogel which could facilitate the repair of damage to the dermal layer. The release of calcium from the alginate was shown not to affect keratinocyte maturation, which allowed the keratinocytes to form a stratified layer in which the cells did not all terminally differentiate and remained viable. The keratinocytes could therefore repopulate the wound site in clinical application and provide wound closure. Alginate hydrogel is potentially an effective scaffold for the co-culture and delivery of keratinocytes and fibroblasts to a wound bed to provide wound closure and expedite the healing process.

Keywords: Alginate, Tissue Regeneration, Wound Healing, Keratinocytes, Fibroblasts.

## 1. INTRODUCTION

### *1.1 Skin and Wound Healing*

Skin consists of two main layers known as the epidermis and the dermis, in which the main cell types are the fibroblast and the keratinocyte. Chronic wounds are wounds that do not heal in an orderly set of stages and in a predictable amount of time the way most wounds do (Supp and Boyce, 2005). These wounds are usually full-thickness and therefore lack both a dermal and epidermal component. The paracrine interactions between the fibroblasts and keratinocytes have been found to be essential for effective wound healing, and therefore it is important that both cell types are present within the tissue engineered skin (Spiekstra et al., 2007). Although a number of tissue engineered skin constructs exist, some simply aim to replace the epidermal layer and thus only provide wound closure but not necessarily structural integrity. The constructs that aim to replace the dermal layer generally contain animal derived components such as collagen or de-epidermalised dermis (DED) which can present risks of disease transmission and immunological rejection. There is a significant need for a tissue engineered bilayer which contains no animal derived components (MacNeil, 2007).

### *1.2 Tissue Engineering*

There is a considerable demand for the development of novel methods to replace diseased or damaged tissues. Synthetic materials have been used as tissue replacements for many centuries, but issues remain surrounding the integration of these materials into the body. Tissue engineers seek to produce and condition tissues *in vitro* with the intention of implanting the resulting construct into the body (Langer and Vacanti, 1993). In order to mimic the 3D organisation of cells in a range of tissues they are typically seeded onto a scaffold composed of a macro-porous sponge-like material. Although such structures provide a certain amount of 3D organisation, the cells are still adhering to a 2D surface. It has been demonstrated that cells that cultured on 2D substrates exhibit distinct phenotypes when compared with cells in the extracellular matrix (Pedersen and Swartz, 2005). In order to produce a tissue representative of that found *in vivo* it is therefore desirable to culture the cells in an environment that mimics

both the structure and spatial resolution of the extracellular matrix (ECM). Hydrogels represent a class of biomaterials which provide such a 3D environment to encapsulated cells (Boontheekul et al., 2005).

### *1.3 Alginate*

Alginate is a polysaccharide isolated from sea weed which can be crosslinked with calcium to form a hydrogel. The highly porous structure of alginate hydrogel allows for good mass transport properties so that nutrients, waste products and signalling molecules can effectively perfuse the scaffold to maintain cell viability (Rowley et al., 1999). Alginate has been used for medical applications such as for drug encapsulation for slow and sustained release of drugs, microencapsulation of cells to protect them from immunological responses when implanted in the body, as a filler to provide bulk to areas where tissue has been lost and in wound dressings (Augst et al., 2006; Boateng et al., 2008). In addition it has been investigated as a material for tissue engineering bone, cartilage and other materials due to its mild gelling reaction, lack of toxicity and its approval for medical applications (Drury and Mooney, 2003; Smith et al., 2007).

In the present study alginate hydrogel was investigated as the scaffold for delivery of a fibroblast/ keratinocyte co-culture to the wound bed to facilitate replacement of the dermis and epidermis. 3T3 fibroblasts and primary rat keratinocytes were used as models for autologous fibroblasts and keratinocytes.

## 2. METHODS AND MATERIALS

### *2.1 Materials and Chemicals*

All chemicals were purchased from Sigma Aldrich (UK) unless otherwise specified.

### *2.2 Fibroblast Encapsulation*

5% w/v low viscosity alginate (20-40 centipoise (cps) for 2% w/v at 25°C) was sterilised by autoclave then used to encapsulate NIH 3T3 cells (LGC, Middlesex, UK) at density of $7.5x10^5$cells/ml. The alginate/3T3 dispersion was added dropwise into a bath of 100mM $CaCl_2$ and left to incubate at 37ºC for 2h to form cross linked spheres of 3.0±0.2 mm diameter, then washed three times in non-supplemented DMEM. Acellular samples for ICP-MS were prepared in the same manner. To form an alginate encapsulating cell sheet, the suspension was used to cover the surface of a petri-dish and immersed in 100mM $CaCl_2$ for 2h to allow for complete cross-linking of the alginate. The displacement of alginate by calcium chloride was prevented by covering the alginate hydrocolloid with a layer of filter paper impregnated with 100mM $CaCl_2$. Cells were grown and maintained in high glucose DMEM supplemented with 10% foetal bovine serum (FBS) (PAA, Somerset, UK), 1% penicillin-streptomycin (P/S), 2.25% HEPES and 2% L-glutamine. All cultures were maintained at 37ºC with 5% $CO_2$ and 100% relative humidity and media was changed three times weekly.

### *2.3 Keratinocyte cultures*

Keratinocytes were obtained from neonatal rat sacrificed by cervical dislocation. The keratinocytes were isolated using 0.25% trypsin± (7\$DQG PHFKDQLFDO GLVVRFLDWLRQ DQG FXOWXUHG LQ 0(0ÐP¶ ) supplemented with 50mM hydrocortisone, 10µg/ml insulin, 25ng/ml EGF, 10% FBS (PAA), 1% P/S, 2.25% HEPES, 2% L-glutamine as monolayers, with the support of a 3T3 feeder layer prepared by treatment with 4mM Mitomycin C for 2h at 37ºC, until sufficient number of keratinocyte cells were obtained, as determined by counting using a haemocytometer. The keratinocytes were seeded at a density of $0.2\times10^6$ cells/$cm^2$ onto the surface of alginate/ fibroblast cell sheet or Thincert TM cell culture inserts, in the presence of a feeder layer (control). After 7 days submerged culture, the keratinocytes were raised to the air/liquid (A/L) interface for 7 days for evaluation by immunohistochemistry (IHC) and 4 days at the A/L interface for evaluation by the reverse-transcription polymerase chain reaction (RT-PCR) and live/ dead staining. All keratinocyte cultures were maintained at 37ºC with 5% $CO_2$ and 100% relative humidity and media was changed three times weekly.

*2.4 Fibroblast Growth*

A stock solution of 5mg/ml MTT in phosphate buffered saline was added to supplemented DMEM at a concentration of 10% (v/v). Samples of alginate/ 3T3 beads were removed from the media, washed in DMEM and immersed in the resulting 10% solution of MTT for 18hrs for growth analysis. The MTT solution was then removed and replaced with HCl-isopropanol and left for 2.5hrs to allow the formazan produced to dissolve and diffuse out of the alginate. The optical absorbance of the solution at 620nm was measured using a spectrophotometer (Cecil, Cambridge, UK). This amount of formazan produced by cellular reduction of MTT was determined from the absorbance at 620nm according to a calibration of different cell concentrations in alginate, as determined by counting on a haemocytometer, and the absorbance at 620nm after application of the MTT assay.

*2.5 Inductively coupled plasma - mass spectroscopy (ICP-MS)*

1ml samples of 5% w/v alginate beads were prepared and immersed in 4ml supplemented DMEM. The samples were prepared and maintained according to the method described for encapsulated fibroblasts. Before assaying the calcium concentration the media was unchanged for 4 days. The calcium ion concentrations in the culture medium was determined using ICP-MS (Varian,Darmstadt, Germany) against standard solutions of 50 ppm and 100 ppm (Merck, Darmstadt, Germany). Samples were performed in triplicate at days 0, 6, 8 and 20 and the mean±SD is shown at each time point.

*2.6 Immunohisochemistry (IHC)*

Samples were fixed in formalin buffer for 24 h before progressive dehydration in cassettes (Thermo Shandon, Citadel 1000, Cheshire, UK). The dehydrated samples were then embedded in paraffin wax (Sakura, Tissue Tek, TEC) and stored at room temperature. Sections of 5µm were taken using a rotary microtome (Leica RM 2035) before attachment to Superfrost glass slides (Laboratory Sales (UK) Limited, Rochdale, UK) and dried at 56°C for 2h before removal of the wax in xylene and staining with Pan Cytokeratin (Pan K) labelled with diaminobenzidine (DAB) and Mayers Haematoxylin (Surgipath Europe Ltd, Peterborough, UK) according to the manufacturers instructions to label cytokeratins brown and cell nuclei blue black, respectively. Coverslips were then applied to the slides using XAM (BDH Laboratory Supplies, Poole, UK) and viewed with a light microscope.

*2.7 Reverse Transcription Polymerase Chain Reaction (RT-PCR)*

Keratinocyte layers were removed from the alginate/ fibroblast dermal analogue and control scaffolds using 0.25% trypsin±0.02% EDTA. The trypsin was inactivated by addition of supplemented media before centrifugation to form cell pellets. mRNA was isolated from the cell pellets using a RNeasy Mini Kit (Qiagen, Crawley, UK). Reverse transcription of RNA to cDNA was performed using an Omniscript RT Kit (Qiagen, Crawley, UK). Polymerase chain reactions were performed using a red TAQ ready mix PCR mix and resulting products were detected by agarose gel electrophoresis, stained with ethidium bromide and visualised under UV illumination. GAPDH expression was used for normalisation (Invitrogen, Paisley, UK) and the target genes analysed were involucrin and keratin 1, 5 and 13 (Invitrogen, Paisley, UK).

*2.8 Live/ dead staining*

Sections of 1mm thickness were taken from the centre of alginate beads containing 3T3 cells 4 days post encapsulation, using a razor blade and keratinocytes were stained *in situ* on the surface of the alginate/ 3T3 dermal analogue. Samples were immersed in 0.2µg/ml Calcein acetoxymethylester for 15 mins and 2.5µg/ml Propidium Iodide (Invitrogen, Paisley, UK) for 5 mins in DMEM at 37ºC to stain live cells green and dead cells red, respectively, when visualised with a fluorescence microscope.

## 3. RESULTS

Encapsulated fibroblasts were shown to be evenly dispersed and remain viable throughout the 3mm diameter of the alginate hydrogel bead after 4 days encapsulation (Fig. 1A). The effect of long term encapsulation of fibroblasts in

5% w/v alginate hydrogel on growth was assessed over an 18 day period by cell counting using the MTT assay (Fig. 1B). The number of viable fibroblasts present within the alginate hydrogel did not deviate significantly from $0.44\times10^6$ cells over the duration of the study, demonstrating that the fibroblasts were in a quiescent state within the hydrogel matrix.

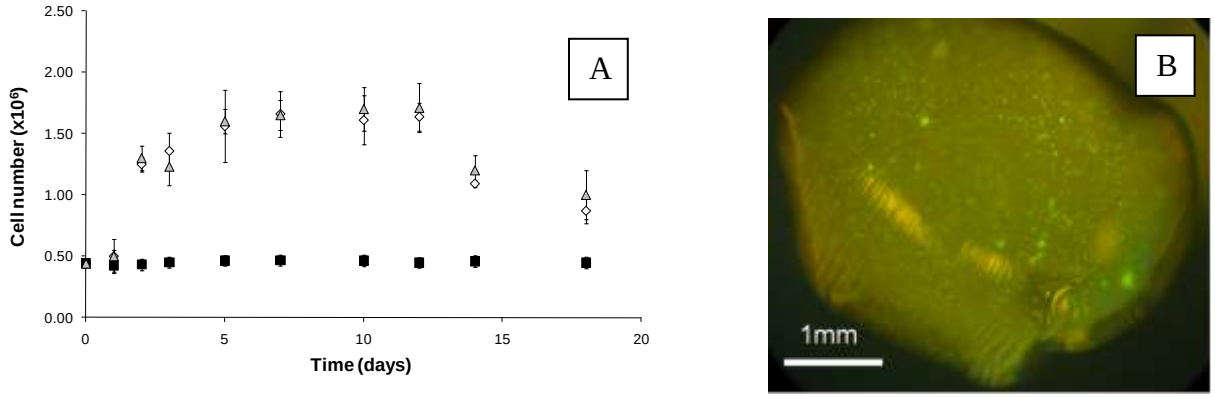


Fig. 1. A. Encapsulated fibroblasts (black squares) were shown to remain at a constant cell number for 18 days. When keratinocytes were released from alginate (grey triangles) these cells were shown to return to normal growth, as seen before the fibroblasts were encapsulated (open diamonds). B. Live/ Dead stained encapsulated fibroblasts (day 4) showed that cells remain viable throughout the diameter of the bead and the cells were evenly dispersed.

The alginate hydrogel scaffold degraded over time by the release of calcium cations which cross-link the structure into the culture medium (Fig. 2). The concentration of calcium in the medium increased between day 0 and 6 then gradually fell between day 6 and 20, indicating that the rate of degradation decreases with time (Fig. 2). The calcium release profiles for acellular and fibroblast encapsulating samples were shown to be very similar, indicating that the cells had very little effect on the degradation of the scaffold. Fibroblasts were observed on the surface of the culture dish in which the alginate was incubated, illustrating that the degradation resulted in the release of encapsulated fibroblasts. Fibroblasts that were released from the alginate hydrogel matrix were shown to resume mitotic activity, and demonstrated the normal growth characteristics that are observed with 2D culture (Fig. 1B).

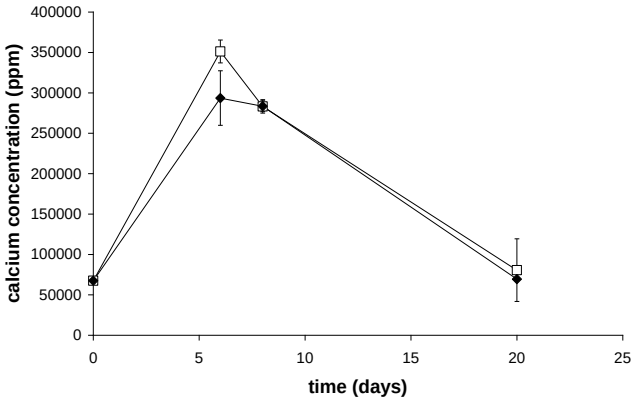


Fig. 2. Change in calcium concentration of cell-culture media with time due release of calcium cations cross-linking the 5% w/v alginate hydrogel. Fibroblast encapsulating samples (closed diamonds) and acellular samples (open squares) show similar calcium release profiles.

A stratified epidermal layer was shown to form on the surface of the alginate after 7 days submerged culture followed by 7 days culture at the A/L interface, as indicated by positive staining for PanK (Fig. 3A). Expression of various proteins specific to different sublayers of the epidermis were assayed by RT-PCR after 4 days culture at the

A/L interface (Table 1). The keratinocyes were shown to express keratin 5, a marker of basal cell lineage, indicating the presence of a basal layer. Expression of keratin 1 and 13 which are specific to the suprabasal epidermal layers was also detected, indicating that some cells had differentiated to form suprabasal layers. Expression of involucrin, a marker of terminal differentiation to form the cornified layer of the epidermis, was not detected indicating that the keratinocytes had not terminally differentiated. The keratinocytes were confirmed to remain viable by the presence of mainly green staining in Fig. 3B. The same pattern of expression of the four proteins was observed for control epidermal layer and the epidermal layer formed upon the surface of the alginate/ fibroblast dermal analogue.

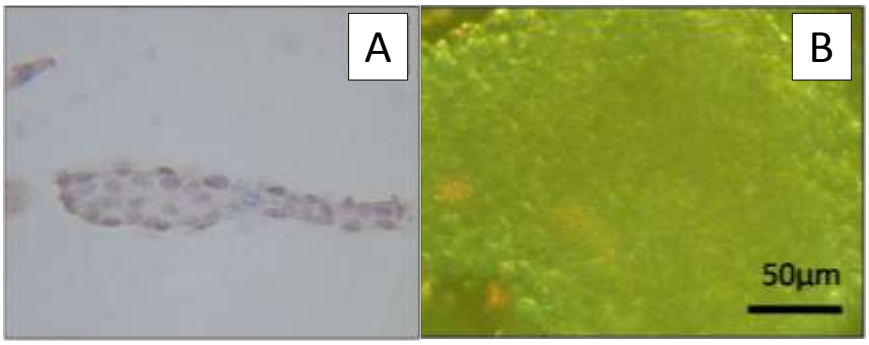


Figure 3. A. A stratified layer formed upon the surface of the alginate/ fibroblast dermal analogue and stained positively with Pan Cytokeratin, indicating that it was formed of keratinocytes and not fibroblasts. B. Live/dead staining of the stratified layer on the alginate/ fibroblast surface showed keratinocytes remained viable.

Table 1. Expression of keratin 5, 13 and 1 and involucrin after 7 days submerged culture and 4 days culture at the air-liquid interface. The keratinocytes grown upon the surface of alginate/ fibroblast dermal analogue expressed keratin 5, 13 and 1 but not involucrin. The same expression pattern was observed for keratinocytes cultured on polymer membranes in the presence of a feeder layer (control).

| | **Control** | **Alginate** |
|---|---|---|
| **Keratin 5** | Expressed | Expressed |
| **Keratin 13** | Expressed | Expressed |
| **Keratin 1** | Expressed | Expressed |
| **Involucrin** | Absent | Absent |

## 4. DISCUSSION

Fibroblast presence is essential to the formation of a full-thickness epidermis (Werner et al., 2007), but the fibroblasts must be mitotically inhibited to prevent over-growth of the keratinocyte/ fibroblast co-culture (Clark et al., 2007). Encapsulation of fibroblasts within the 5% w/v alginate hydrogel matrix was found to effectively inhibit their growth (Fig. 1) in contrast to what has been found for HepG2 liver cells encapsulated at a higher cell density in a lower concentration alginate (Khattak et al., 2006). It is therefore proposed that the lack of cell proliferation in our study is due to the cells being mechanically confined from each other by the alginate scaffold. This growth inhibition allowed for keratinocytes to form a stratified epidermal layer upon the surface (Fig. 2).

Calcium is a key regulator of keratinocyte maturation, and in excess can cause all keratinocytes to terminally differentiate and die (Lansdown, 2002). The alginate hydrogel degraded over time by the release of cross-linking calcium cations into the culture medium (Fig. 2) in a similar manner to that which has previously been observed with other alginate hydrogels (Boontheekul et al., 2005). The release of calcium from the scaffold was found to have no effect on the expression profile of keratinocytes after 4 days at the A/L interface indicating that the release of calcium from the alginate was not significant enough to alter the normal course of keratinocyte maturation (Table 1). After 7 days at the A/L interface the keratinocytes were shown to be viable by live/ dead staining, again illustrating that the keratinocytes had not terminally differentiated due to the calcium released from the alginate hydrogel (Fig.

2b). The keratinocytes should therefore be able to undergo further proliferation and differentiation at the wound site to produce a full thickness epidermis to provide wound closure.

As the alginate scaffold degraded, fibroblasts were observed to be released from the scaffold and attached to the tissue culture plastic of the petri-dish in which the alginate was cultured. The growth of released fibroblasts appeared very similar to that seen before encapsulation (Fig. 1) and therefore the fibroblasts should be capable of replacing the lost dermis at the wound site, after the closure of the wound by the keratinocytes. As the scaffold degrades at the wound site other cell types which are involved in wound healing may also be able to migrate into the scaffold to facilitate wound healing and integration of the tissue engineered scaffold into the body.

## 5. CONCLUSIONS

Alginate hydrogel is a promising scaffold and delivery vehicle for a co-culture of keratinocytes and fibroblast to the wound bed to facilitate the healing of full thickness skin wounds. The keratinocytes could provide wound closure to restore the barrier function of skin. The alginate should degrade with time to release the encapsulated fibroblasts to facilitate replacement of the dermal layer. Further work will seek to evaluate the behaviour of the scaffold *in vivo* when seeded with autologous fibroblasts and keratinocytes, after cultivation in serum-free media to avoid immunological responses.

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# A COMPARATIVE STUDY OF IOTA CARRAGEENAN, KAPPA CARRAGEENAN AND ALGINATE HYDROGELS AS TISSUE ENGINEERING SCAFFOLDS

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## 1 INTRODUCTION

The increasingly aging population and lack of donor tissue has pushed tissue regeneration to the forefront of modern biomedical research and biopolymers traditionally found in the food and pharmaceutical industry are increasingly being used in tissue engineering. The development of tissue engineering provides the opportunity to take a sample of cells from a patient which are then cultured *in vitro* to organise into functional tissue that can then be implanted back into the patient, ultimately, overcoming problems of tissue rejection and the need for donor tissue. To grow replacement tissue requires a suitable substrate or scaffold for cells to attach, proliferate and organise into viable tissue that can be safely implanted into the body. Biopolymers and hydrogel forming biopolymers in particular, have been shown to be a promising scaffold material due having properties that resemble the environment of the mammalian extracellular matrix (ECM). In addition properties such as mild gelation conditions, potential for good mass transport of nutrients and waste molecules, nontoxic nature and biological compatibility provide an advantage over synthetic polymers and ceramic and metallic materials which have also been used as tissue engineering scaffolds.

As well as supporting cell growth and proliferation, the scaffold must have the mechanical characteristics needed to retain its structure for as long as it is needed [1]. Ideally gels used as scaffolds for tissue engineering would have a mild sol-gel reaction that can be controlled at 37°C and at physiological pH, have rapid gelation kinetics and be robust enough for 3D culture and implantation. This explains why much of the research on biopolymers for tissue engineering has been performed on materials such as alginate[2] and chitosan[3] collagen[4] agarose[5] and fibrin[6]. The relative success of the cell culture of the mentioned biopolymers has generated interest in other gel forming biopolymers such as gellan gum[7] and chemically modified gels such as RGD alginate[8]. Performance of the cells when encapsulated in these biopolymers varies due to the cellular interaction with the polymer chemistry and gel network structure.

In this study we have investigated the use of carrageenan as a 3D tissue engineering scaffold for mammalian cells and looked at the impact of structural differences between kappa and iota carrageenan has on the attachment, survival and proliferation of fibroblasts and in comparison with alginate which, is widely used in tissue regeneration. Carrageenan has been used in bacterial culture[9] and has been evaluated as a delivery vehicle for growth factors for mammalian tissue engineering applications[10] however there have been no reports of carrageenan being used exclusively for the 3D culture of mammalian cells.

2 METHODS AND RESULTS

Unless otherwise specified all reagents were obtained form Sigma Aldrich (Poole, UK).

**2.1. Formation of hydrogel scaffolds for encapsulation of fibroblasts**

NIH 3T3 murine fibroblasts were added to 2% (w/v) Na-alginate at a density of 0.75 x $10^6$ cells/ml. The cell-gel suspension was then added drop wise into a bath of 100mM $CaCl_2$ and left to incubate at 37ºC for 2h to form cross-linked spheres of 3.0±0.2 mm diameter.

1% *kappa* and 2% *Iota* carrageenan discs were formed using 3ml autoclaved gel which was cooled to 40°C before a 0.75 x $10^6$ NIH 3T3 murine fibroblast cell suspension was added and pippetted repeatedly to ensure homogenisation. No extra cations were added to the gel solution as there were sufficient salts present within the supplemented media added to cell suspensions to cause the gel to cross-link.

All samples were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% v/v foetal bovine serum (FBS) (PAA, Somerset, UK), 1% v/v penicillin-streptomycin (P/S), 2.25% v/v HEPES and 2% v/v L-glutamine and maintained at 37ºC with 5% $CO_2$ and 100% relative humidity and media was changed three times weekly. Control samples without cell seeding were also prepared for degradation studies.

**2.2 Formation of hydrogel scaffolds for surface seeding**

Alginate discs were prepared using sterile 2% low viscosity Na-alginate. The discs were formed by covering 3ml Na-alginate with 100 mM $CaCl_2$ for 2h in 6-well tissue culture plates. Following crosslinking, NIH 3T3 murine fibroblasts were seeded on the surface of the alginate at a density of 0.44×$10^6$ cells/ml

*Iota* and *Kappa* carrageenan gel cylinders were formed using sterile 2% (w/v) *iota*-carrageenan and 1% (w/v) *kappa* carrageenan in 12-well culture plates containing 3ml of the gel solution per well before addition of 1 ml 200 mM KCl. The cross-linked *kappa* and *iota*-carrageenan discs were then seeded at a cell density of 0.75×$10^6$. The gel discs were topped with supplemented DMEM and maintained at 37ºC with 5% $CO_2$ and 100% relative humidity.

**2.3 Analysis of cell morphology and survival**

Following 10 days in culture, samples of the gels were removed and the cells were analysed using a Live/Dead® Viability/Cytotoxicity Kit. This procedure, based on the uptake of calcein AM and conversion to fluorescent calcein (green) by intracellular esterases in live cells and the uptake of propidium iodide (red) by cells with damaged plasma membranes, was used in conjunction with fluorescence microscopy to assess cellular responses to the alginate and carrageenan scaffolds. The results for the encapsulated samples showed that >95% of cells remained viable in alginate. The rounded morphology, however, also indicate that they were not well attached to the gel (Fig. 1A). Similarly cells encapsulated in *kappa*-carrageenan are rounded in appearance on day 10 (Fig 1B) with > 95% of the cells remaining live. Samples of *iota*-carrageenan (Fig. 1C) also had a >95% survival, however, the appearance of thin projections and overall elongation of the cells suggests attachment of the cells to the hydrogel. It is hypothesised that the lack of ionically bound junction zones within the structure of iota-carrageenan gels provides spaces between the polymer chains that are sufficient in allowing the cells to grow and proliferate similar to the findings of Jeon et. al. (2007)[11]. For surface seeded samples only a few cells were seen to be attached to alginate and *iota*-carrageenan after 10

spontaneously reform below their melting point following mechanical disruption[12] allowing cells to migrate throughout the gel without disrupting the network, unlike in kappa-carrageenan and alginate where the cells are confined within a more tightly bound gel network. After 5 days in culture, this number reduced (from $4.9\times10^6$ to $1.1\times10^6$ by day 12). Proliferation was also seen in *kappa*-carrageenan, however, not at the same rate as in *iota*-carrageenan and it was not until day 10 that the cells in kappa carrageenan were of comparable number to those in the iota-carrageenan.

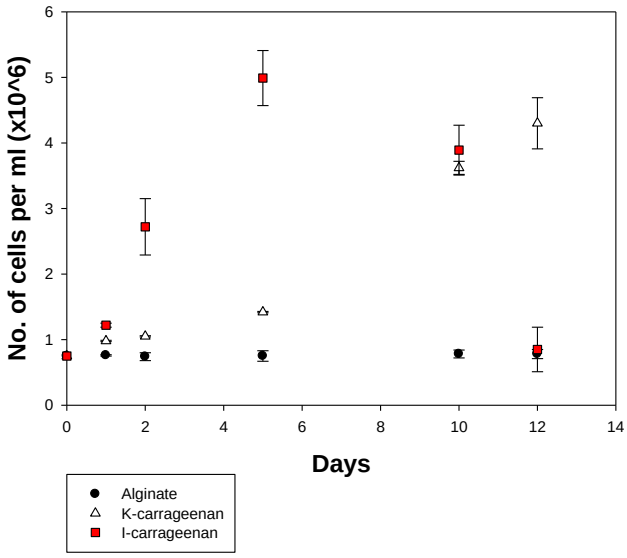


**Fig. 3: Number of viable cells calculated from the absorbance of formazan of the encapsulated cells. Vertical error bars represent the standard deviation of the reported mean values (n=3).**

## 2.4 Degradation

The changes in mass of gels with and without cells was determined by measuring the dry mass of the samples (by slow vacuum drying) on a daily basis in a vacuum freeze-drier (Edwards, EF03, Sussex, UK) for 16h. This was to quantify any fluctuations in mass within the samples which may occur as a consequence of polymer degradation, protein absorption from the culture media or formation of extracellular matrix.

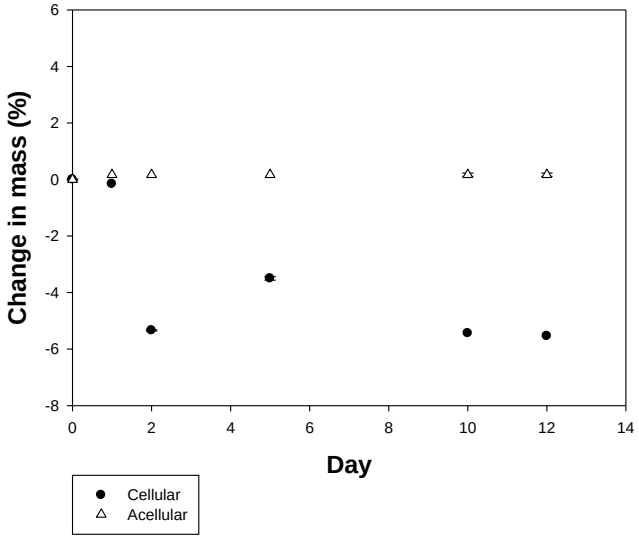


**Fig. 4: Changes in the mass for *Kappa*-carrageenan with and without cells. Vertical error bars represent the standard deviation of the reported mean values (n=3).**

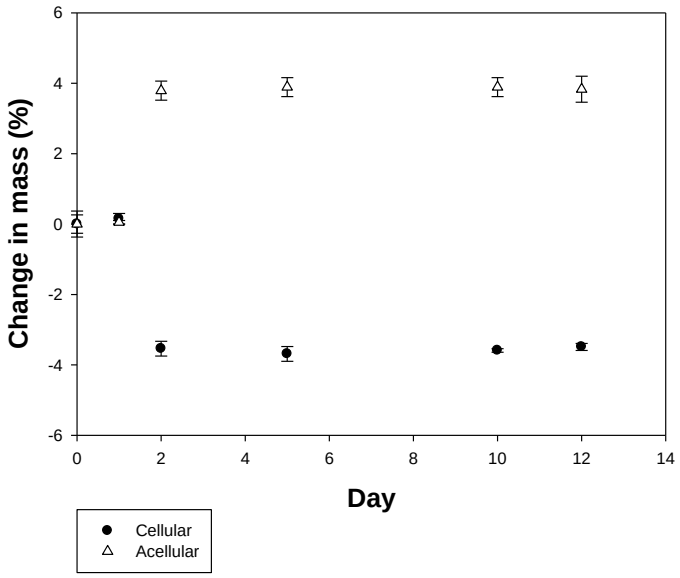


**Fig. 5: Changes in the mass for *Iota*-carrageenan with and without cells. Vertical error bars represent the standard deviation of the reported mean values (n=3).**

The degradation rates of the three gels were found to vary considerably, with no statistical difference seen between the cellular and acellular samples of alginate ($P > 0.05$) (results not shown). The acellular samples of *kappa*-carrageenan remained unchanged for the duration of the study, however, the addition of cells within the gel caused a gradual loss in total mass suggesting degradation of the polymer. The gel mass of acellular samples of *iota*-carrageenan increased by a total of 3.9% 2 days post encapsulation indicating the possibility of protein absorption from the culture media to the carrageenan, enabling cell attachment. This supports the findings from the proliferation assay and the observations of cell attachment. The *iota*-carrageenan containing cells showed a 3.5% reduction in mass which, is thought to be due to degradation of the gel which was visually apparent when samples were being transferred to the freeze dryer for measurements. The kappa carrageenan samples where observed to be more robust than the iota-carrageenan however the gel integrity of both types did reduce through the duration of the study. Kappa-carrageenan was initially observed to be a stronger gel in comparison with iota-carrageenan and interestingly, these physical properties may be the cause of initial cell proliferation observed in iota-carrageenan which were not seen in the kappa form. Eventual dissolution of iota-carrageenan lead to difficulty in quantifying the proliferation of encapsulated cells within the polymer at day 12 which accounts for the reduction in cellular proliferation measured using the MTT assay. The gel network of the kappa-carrageenan samples also began to lose its integrity, however, this was not apparent until day 10 of the study which correlates with a dramatic increase in the proliferation of encapsulated cells. This is in contrast to the alginate samples which appeared unchanged throughout. This demonstrates the necessity of investigating the mechanical properties of hydrogel scaffolds in relation to cellular behaviour.

## 3.0 CONCLUSION

In conclusion we have shown that cells can be encapsulated and remain viable in both kappa and iota carrageenan for up to 12 days which is comparable with alginate which is a widely used tissue engineering substrate. There was no evidence of attachment of cells to the alginate or the kappa carrageenan however some attachment was evident when using

iota carrageenan as the scaffold for both encapsulated cells and surface seeded cells. It was also shown that proliferation of the cells within the scaffolds occurs at different rates possibly due to the mechanical properties and network architecture of the gels. Fluctuations in mass were also measured in the scaffolds which correlated with changes in cellular proliferation.

Subsequently, this study has highlighted the need for investigations into the mechanical properties of hydrogels when applied as cell scaffolds. The potential for utilising biopolymers and mixtures of biopolymers traditionally used in the food and pharmaceutical industry for this purpose is apparent as the subtle differences in gel architecture can have a dramatic effect on cell culture. This preliminary study suggests that both kappa and iota-carrageenan may be suitable hydrogels for use as a scaffold for tissue engineering purposes and as a tool for examining cells as proliferation occurs. Iota-carrageenan in particular, may have applications as a cell delivery vehicle due to the initial proliferation and subsequent dissolution. Furthermore, chemical modifications, and the development of polymer blends to change the structure and gelation behaviour may be of use to tailor the scaffolds further.

**Acknowledgements**
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