Development of poly(vinylpyrrolidinone) networks for treatment of skin graft contracture.

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Abstract.

Skin graft contracture is a problem facing many burns patients treated with skin grafts. Currently grafts under suspicion of contraction are treated with pressure garments to prevent contractures progressing. Patients may have to wear these for many months however once contractures have formed surgical intervention is commonly required.

The aim of this project was to develop a hydrogel drug delivery system to prevent or reduce skin graft contracture. Two potential anti-contraction agents, identified based on prior work from our laboratory were initially examined for their effectiveness in preventing contracture formation using two 3D models of contraction - collagen I gels and a reconstructed human skin model. β-aminopropionitrile (βAPN), a non-competitive lysyl oxidase inhibitor significantly reduced contraction in the reconstructed skin model but not the collagen gel model while 4-methyl umbelliferone (4-MU) reduced contraction in both models.

For hydrogel drug delivery poly(N-vinylpyrrolidinone) (PNVP) hydrogels were developed with material properties suitable for use as a non-cytotoxic wound dressing. Two crosslinked PNVP's were investigated, one crosslinked with ethylene glycol dimethacrylate (EGDMA) and the other crosslinked with diethyleneglycol bis allylcarbonate (DEGBAC). The different crosslinkers led to hydrogels with different mechanical and slightly different biological properties. Although neither hydrogel proved to be suitable for culturing cells on, indirect contact with both showed them to be biocompatible and in some cases stimulatory to fibroblasts. These hydrogels were evaluated for their uptake and release of βAPN and 4-MU. Hydrogels were then used to deliver βAPN and 4-MU to reduce skin cell contraction in both collagen gels and the reconstructed skin model with some promising preliminary results showing hydrogels releasing 4-MU to reduce contraction in the 3D collagen gel model.

Contents.

Acknowledgements.

Abbreviations.

4-MU	4-methyl umbelliferone
5-FU	5-Fluorouracil
AA	Acrylic acid
AIBN	α,α'-azobisisobutyronitrile
ALI	Air-liquid interface
ATRP	Atom transfer radical polymerisation
BDTB	Benzene dithiobenzoate
BSA	Bovine serum albumin
BSE	Bovine spongioform encephalitis
cAMP	Cyclic adenosine monophosphate
CEA	Cultured epithelial autograft
CJD	Creutzfeld Jacob disease
DAPI	4',6-Diamidino-2-phenylindole
DED	De-epithelised acellular dermis
DEGBAC	Diethyleneglycol bisallylcarbonate
DEGDMA	Diethyleneglycol dimethacrylate
DEJ	Dermo-epidermal junction
DMA	Dynamic mechanical analysis
DMEM	Dulbecco's modified eagles medium
DMF	Dimethylformamide
DMSO	Dimethyl siloxane
DSC	Differential scanning calorimetry
E	Elastic modulus or Young's modulus
ECM	Extracellular matrix
EGDMA	Ethylene glycol dimethacrylate
EGF	Epidermal growth factor
ESTA	Eluted stain assay
EtOH	Ethanol
EWC	Equilibrium water content
FCS	Foetal calf serum
FDA	Food and drug administration
FGF	Fibroblast growth factor
FTIR	Fourier transform infrared
FTSG	Full thickness skin graft
G	Shear modulus
GAG	Glycosaminoglycan
GMA	Glycerol methacrylate
GRGDS	Glycine-Arginine-Glycine-Aspartic Acid-Serine
H&E	Haematoxylin and Eosin

HA	Hyaluronan
HAS	Hyaluronan synthase
HB-EGF	Heparin binding epidermal growth factor
HCEC	Human corneal epithelial cells
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
IFNγ	Interferon γ
IgG	Immunoglobulin G
IPA	Propan-2-ol
kDa	Kilo Dalton
KGF	Keratinocyte growth factor
MMP	Matrix metalloproteinase
MPa	Mega Pascal
MPTMSE	1-methoxyphenyl-1-(trimethylsilyloxy)ethylene
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H-tetrazolium
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
mV	Milli volt
Mw	Average molecular weight
n	Diffusional exponent
NMR	Nuclear magnetic resonance
NVP	N-vinyl pyrrolidinone
PAA	Poly(acrylic acid)
PAAm	Poly(acrylamide)
PBS	Phosphate buffered saline
PBT	Poly(butylene terepthalate)
PCL	Poly(caprolactone)
PDGF	Platelet derived growth factor
PEG	Poly(ethylene glycol)
PEGT	Poly(ethylene oxide terepthalate)
PEO	Poly(ethylene oxide)
PET	Poly(ethylene terepthalate)
PGA	Poly(glycolic acid)
PHEMA	Poly(hydroxyethylmethacrylate)
PLA	Poly(lactic acid)
PNA	*Para*-nitroaniline
PNaAMPS	Poly(2-acrylamido-2-methyl-1-propanesulfonic sodium)
PNaSS	Poly(sodium p-styrene sulfonate)
PNIPAAm	Poly(N-isopropylacrylamide)
PNVP	Poly(vinyl pyrrolidinone)
PPH	Propranolol hydrochloride
PPO	Poly(propylene oxide)
PTFE	Poly(tetrafluoroethylene)
PVA	Poly(vinyl alcohol)
PVP-I	Poly(vinylpyrrolidinone)-iodine

RAFT	Reversible addition fragmentation transfer
RGD	Arginine-Glycine-Aspartic Acid
SDS	Sodium dodecyl sulphate
SEC	Size exclusion chromatography
SEM	Standard error of mean
sHA	Sulphated hyaluronan
STSG	Split thickness skin graft
TCP	Tissue culture polystyrene
Tg	Glass transition temperature
TGF-α	Transforming growth factor α
TGF-β	Transforming growth factor β
THF	Tetrahydrofuran
TNBS	2,4,6-trinitrobenzenesulfonic acid
TNFα	Tumour necrosis factor α
UCS	Ultimate Compressive Strength
VEGF	Vascular endothelial growth factor
WHO	World health organisation
Wt%	Weight %
αSMA	α smooth muscle actin
βAPN	β-aminopropionitrile
βAPN.F	3-aminopropionitrile fumarate
ε	Strain
σ	Stress

Publications.

Papers

L. Smith, C.A. Harrison, S. Rimmer, S. MacNeil. The effect of poly(vinylpyrrolidinone) hydrogels loaded with β-aminopropionitrile and 4-methyl umbelliferone on contraction of collagen gels and tissue engineered skin. In preparation

L. Smith, S. Collins, Z. Liu, S. MacNeil, R. Williams, S. Rimmer. Synthesis and properties of functional poly(vinylpyrrolidinone) hydrogels for drug delivery. *ACS symposium series*, in press. Invited paper

L. Smith, S. Rimmer, S. MacNeil. Examination of the effects of poly(vinylpyrrolidinone) hydrogels in direct and indirect contact with cells. *Biomaterials*. 27(2006) 2806-2812

Published abstracts

L. Smith, S. MacNeil, S. Rimmer. Synthesis and characterisation of a poly(vinylpyrrolidinone) hydrogel for use in burns treatment. *PMSE preprints*. 95(2006)

L. Smith, S. Rimmer, S. MacNeil. Development of Poly(N-vinylpyrrolidinone) hydrogels for treatment of skin graft contracture. *Eur. cell. mater.* 11 suppl.3,2006 (66)

1. Introduction.

In the UK approximately 250,000 people suffer burn injuries per annum of these 175,000 attend an accident and emergency department. 13,000 people are subsequently admitted to hospital. 1000 patients have burns severe enough to require fluid resuscitation and 50% of these are children under 12 years. On average 300 deaths occur per annum from burns (Hettiaratchy and Dziewulski 2004a). Globally more than 6 million patients require grafts for burn injuries per annum. More than 30% of these will subsequently suffer hypertrophic scarring and graft contracture (Harrison and MacNeil Submitted for publication).

Skin graft contracture is a major clinical problem associated with healing of grafted burns patients. Over the past 15 years the group of Professor Sheila MacNeil at the University of Sheffield have been investigating the causes of skin graft contracture, developing *in vitro* models and investigating methods of blocking skin graft contracture.

1.1 Skin morphology.

Before introducing skin graft contracture it is necessary to briefly introduce skin, its structure and function, the normal wound healing process and what happens when wounds do not heal correctly.

Skin is often referred to as the largest organ in the body having a surface area of 1.5-2 m^2. It has many functions, sensing its surroundings i.e. sensing touch and temperature; it also provides a protective barrier against environmental insult i.e. ultraviolet radiation and pathogens (Marte, Finkelstein et al. 2007). One of the more important aspects of the skins barrier function is the control skin exerts over the loss of water from the body (Paranteau, Hardin-Young et al. 2000). Skin is split into two main layers the epidermis and dermis. A schematic diagram showing the gross morphology of skin is shown in figure 1.1.
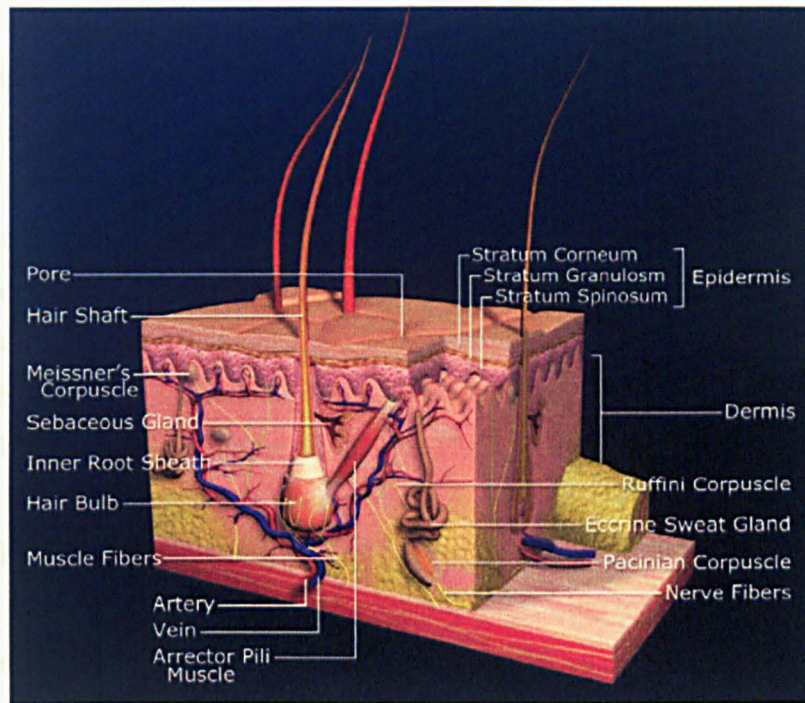
Figure 1.1 Schematic diagram of skin obtained from http://www.wikipedia.org.

The epidermis presents a physical barrier between the body and the external environment. It is composed primarily of keratinocytes forming a stratified squamous epithelium and is subdivided into four further layers the stratum corneum, stratum granulosum, stratum spinosum and stratum basale. Proliferative cells are found in the stratum basale, and anchor the epidermis to the dermis. These cells continually replenish the cells of the epidermis. As cells move upwards towards the surface of the epidermis they terminally differentiate until they integrate into the stratum corneum. This is a layer of dead cells that provides the physical barrier of the skin. This outermost layer is continually being sloughed off the surface of the skin. As cells progress upwards from the stratum basale they stop being proliferative, Keratin filaments and desmosomes increase the strength and integrity of the intermediate layers (stratum granulosum and stratum spinosum). During the later stages of differentiation lipids are extruded into the intercellular space. The nuclei and organelles inside the cells breakdown and a highly crosslinked protein envelope is formed inside the cell membrane. This envelope is connected to a network of intracellular keratin filaments increasing the strength of the epidermis. In addition to keratinocytes which are the main cell population in the epidermis, Langerhans' cells;

dendritic cells of the immune system, melanocytes; which produce and distribute melanin to neighbouring keratinocytes, and merkel cells; which act as sensory receptors can be found (Paranteau, Hardin-Young et al. 2000).

Between the epidermis and dermis is the basement membrane. This is not visible to the naked eye or even under a light microscope. It is a specialized complex arrangement of extracellular matrix (ECM) proteins, namely collagen IV, VII and laminin that attaches epithelial cells to the underlying dermal matrix (Ghosh, Boyce et al. 1997; Ralston, Layton et al. 1999). The basement membrane appears to be produced by both fibroblasts and keratinocytes. It promotes keratinocyte attachment, proliferation and differentiation (MacNeil 1994).

Below the basement membrane is the dermis. As the epidermis can be divided into regions so can the dermis. The papillary dermis, directly below the epidermis and the reticular dermis directly below that see figure 1.2 below. The upper surface of the papillary dermis forms ridges which interlock with similar ridges on the base of the epidermis; these ridges are called Rete ridges. The main difference between the papillary dermis and reticular dermis is the structure of the collagen bundles found there. The papillary dermis contains loose bundles of collagen whilst the reticular dermis contains dense bundles of collagen and elastin and it provides skin with its overall strength and elasticity. Within the dermis are many different inclusions, hair follicles, sebaceous glands, sweat glands etc. The dermis also contains blood vessels, nerves and lymphatic vessels The primary cell population in the dermis is the fibroblast which secretes large amounts of collagen, they can secrete enzymes such as collagenases and other proteases that can 'remodel' the dermis (Ramos-E-Silva and Ribeiro De Castro 2002). Other cells found in the dermis include endothelial cells which are found lining blood vessels, mast cells which contain granules rich in histamine and heparin and are involved in inflammation, macrophages and lymphocytes.
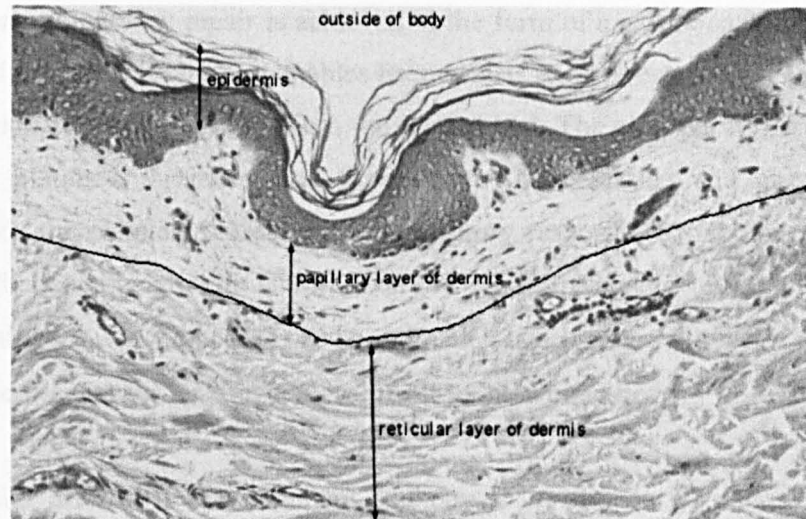
Figure 1.2 Histology slide illustrating the epidermis, papillary dermis and reticular dermis. Image obtained from http://www.bu.edu/histology/p/082031oa.htm.

1.2 Wound healing.

As wound healing is a complex process involving multiple cell types, growth factors, cytokines, peptides etc. and many different processes a brief overview will be given here. There are many factors that affect the wound healing process especially in major wounds such as burns, age, nutrition, infection, associated illness and cytotoxic treatments all impact in either a positive or negative way. Wound healing is traditionally divided into three phases, inflammation, proliferation and remodelling or maturation (Broughton, Janis et al. 2006). These three phases of wound repair are not mutually exclusive but rather overlap in time (Clark 1995). The primary goals of wound treatment are rapid wound closure and a functional and aesthetically satisfactory scar. Recent advances in cellular and molecular biology have expanded the body of knowledge of the biological processes involved in wound healing and tissue regeneration and have led to improvements in wound care (Singer and Clark 1999).

1.2.1 Inflammation.

The skin serves as a protective barrier against the outside world. Any break in this barrier must therefore be mended rapidly. Most cutaneous wounds result in trauma to the blood vessels in the dermis, but this is not always the case. If haemorrhage does

occur then a temporary repair is achieved in the form of a clot or scab that plugs the defect (Martin 1997). This clot enables haemostasis to be re-established and provides ECM which aids cell migration into the wound bed. The clot that forms is made of collagen, platelets, thrombin, and fibronectin. The cytokines and growth factors released by the platelets initiate the inflammatory response (Broughton, Janis et al. 2006). Chemotactic growth factors released by the platelets in the clot such as transforming growth factor-β (TGF-β) and platelet derived growth factor (PDGF) are among the triggers for leukocyte recruitment to the wound bed.

Neutrophils are the most abundant leukocytes in the early stages of healing (Mutsaers, Bishop et al. 1997). Activation of inflammatory cells is important, activated macrophages mediate angiogenesis (the formation of new blood vessels), fibroplasia (formation of fibrous tissue) and synthesis of nitric oxide. Neutrophils clear the wound of invading bacteria, cellular debris and non-viable tissue. Neutrophils can generate oxygen free radicals and secrete proteases with either broad specificity i.e. elastase, or matrix metalloproteinase's (MMP's) which have greater specificity. Both of these destroy the existing wounded ECM, protease inhibitors protect unwounded ECM but this protection can be overwhelmed and penetrated if the inflammatory response is extremely robust from a massive release of proteases (Broughton, Janis et al. 2006). Neutrophils not only clear the wound bed of wounded ECM but clear the wound bed of invading bacteria. They are also a source of pro-inflammatory cytokines that serve as some of the earliest signals to activate local fibroblasts and keratinocytes. Unless a wound is grossly infected, the neutrophil infiltration ceases after a few days, expended neutrophils are then phagocytosed by invading macrophages (Martin 1997).

Macrophages are vital for normal wound healing and appear to have a pivotal role in the transition between inflammation and repair (Singer and Clark 1999). They act with neutrophils to phagocytose wound debris and invading bacteria and other pathogenic microorganisms. They are also a source of growth factors and cytokines such as PDGF, TGF-$\beta_{1,2\&3}$, transforming growth factor-α (TGFα), heparin binding epidermal growth factor (HB-EGF), fibroblast growth factors 1,2 and 4 (FGF$_{1,2\&4}$),

vascular endothelial growth factor (VEGF) and tumour necrosis factor-α (TNFα) (Martin 1997; Mutsaers, Bishop et al. 1997).

1.2.2 Proliferation.

The proliferation phase or tissue formation phases contains epithelialisation, angiogenesis and provisional matrix formation (Broughton, Janis et al. 2006). To aid keratinocyte migration MMP secretion is up regulated in migrating keratinocytes and those close to the wound edge. MMP's up regulated include MMP-9 (gelatinase B), MMP-1 (interstitial collagenase) and MMP-10 (stromelysin-2), these quickly start to remove wounded tissue and clotted blood from the wound bed (Martin 1997; Singer and Clark 1999). Keratinocyte growth factor (KGF) is synthesised and secreted by fibroblasts in close proximity to the wound bed. This and the presence of HB-EGF and other growth factors such as TGFα and FGF stimulates keratinocytes to migrate into the wound bed in a leapfrog like fashion (Clark 1995). Keratinocytes migrate from the margins of the wound and the linings of any dermal inclusions i.e. hair follicles within the wound bed. Keratinocytes immediately behind the migrating keratinocytes begin to proliferate but not differentiate and as reepithelialisation progresses basement-membrane proteins reappear in an ordered fashion from the margin of the wound inward (Clark 1995; Martin 1997; Singer and Clark 1999; Broughton, Janis et al. 2006). Once the wound surface has been covered by a monolayer of keratinocytes migration ceases, this is probably caused by contact inhibition. From this point on a new stratified squamous epithelium complete with basement membrane is established from the wound margin inward. Coincident with the synthesis of the basal lamina (the uppermost portion of the basement membrane), keratinocyte MMP expression is 'shut off', and new hemidesmosomal adhesions to the basal lamina reassemble (Martin 1997).

Angiogenesis or neovascularisation is the formation of new blood vessels within the healing wound and it is necessary to sustain the newly forming granulation tissue. Many factors are implicated in angiogenesis the release of growth factors such as FGF$_2$, TGFβ, and VEGF at the wound margin, and low oxygen tension and elevated lactic acid promote angiogenesis (Martin 1997; Singer and Clark 1999; Broughton, Janis et al. 2006). Angiogenesis is a complex process that relies on an appropriate

ECM in the wound bed as well as phenotype alteration, stimulated migration, and mitogenic stimulation of endothelial cells (Clark 1995). Proliferating microvascular endothelial cells both adjacent to and within the wound bed deposit fibronectin and begin to form new capillary tubes (Singer and Clark 1999; Broughton, Janis et al. 2006). However once the wound is filled with granulation tissue angiogenesis ceases and many of the new blood vessels disintegrate as a result of apoptosis (Singer and Clark 1999).

Fibroplasia consists of granulation tissue components that arise from fibroblasts (Clark 1995) and provides provisional support for the migrating epithelium (Linares 2002). Granulation tissue begins to invade the wound approximately four days after injury. The molecules of newly formed ECM contribute to granulation tissue formation by providing a scaffold for cell migration into the wound. These molecules include fibrin, fibronectin and hyaluronic acid (Singer and Clark 1999). Skin fibroblasts are normally sessile and quiescent, but shortly after wounding they become activated (Grinnell 1994). Fibroblasts synthesise a matrix comprising mainly of collagen. In uninjured skin collagen 80-90% of the collagen is collagen I with 10-20% collagen III. In granulation tissue collagen III comprises 30% of the total collagen (Broughton, Janis et al. 2006).

1.2.3 Wound remodelling.

Clinically, the maturation and remodelling phase is perhaps the most important (Broughton, Janis et al. 2006). The main features of this phase are the deposition and organisation of ECM in the wound bed, cell maturation and cell apoptosis (Clark 1995). This third phase overlaps with tissue formation but can proceed for months after the wound appears to be healed. Remodelling of the ECM and maturation of the neoepidermis, fibroplasia and neovasculature begin at the wound margin while granulation tissue is still invading the wound space in all but the smallest of wounds (Clark 1995). Remodelling of the collagen meshwork in the wound bed includes increased crosslinking, breakdown activity of collagenases, decrease in glycosaminoglycans, regression of the capillary neovasculaturity, maintenance of an adequate I:III collagen ratio, and reorientation of collagen fibres in response to mechanical stress (Linares 2002). The degradation of collagen in the wound is

controlled by MMP's secreted by macrophages, epidermal cells and endothelial cells as well as fibroblasts (Singer and Clark 1999). Along with efforts to reepithelialise the wound spatial reduction in the size of the wounded area is also attempted. This occurs by means of centripetal movements of the surrounding skin and shrinkage of the wound contents (Linares 2002). Wound contraction is the relatively rapid mechanical reduction in the size of a wound (Greenhalgh 2002). Any significant loss of dermis tends to contract and distort the skin producing a scar (Ramos-E-Silva and Ribeiro De Castro 2002). Collagen remodelling during the transition from granulation tissue to scar is dependent on continued synthesis and catabolism of collagen at a low rate (Singer and Clark 1999). Clinically, the original redness, elevation, and firm consistency of the new scar tissue gradually evolves into a pale, flat, soft scar tissue which is level with the adjacent skin surface. This phase of 'maturation' includes a gradual replacement of the original scar tissue over a period of at least six months. A resulting scar may be depressed with respect to the normal skin level, show hypotrophic or even atrophic tissue features, be darker or lighter than the surrounding skin, or become hypertrophic (Linares 2002). Healed wounds never attain the same breaking strength as uninjured skin, at one week, the wound only has 3% of its final strength; at three weeks it is 30% and at three months and beyond it is approximately 80% (Singer and Clark 1999; Broughton, Janis et al. 2006). Van Zuijlen et al. (2003) looked to see if there was any difference in the collagen morphology or collagen orientation in normal skin and in scar tissue especially in scars around joints. Despite their original hypothesis that collagen bundles align in a parallel fashion in the direction of mechanical tension during scaring, no difference could be found between collagen orientation for joints and control areas with respect to the mechanical forces acting on the skin. They concluded therefore that mechanical tension caused by joint mobility does not affect collagen structure and subsequently questioned whether tensional load is a causative factor for wound healing complications, such as contracture. However the collagen morphology of scar tissue differed significantly from that of normal skin, as collagen bundles of scar tissue were orientated in a more parallel fashion with respect to action of the mechanical forces than in normal skin (van Zuijlen, Ruurda et al. 2003).

1.2.4 Wound contraction, contracture and the myofibroblast.

Wound contraction is often attributed to the presence of myofibroblasts in granulation tissue. The myofibroblast phenotype is characterised by large bundles of α smooth muscle actin containing microfilaments disposed along the cytoplasmic face of the plasma membrane of the cells, and by cell-cell and cell-matrix linkages (Martin 1997; Singer and Clark 1999). Myofibroblasts are also characterised by an indented nuclear envelope and well developed stress fibres (Scott, Ghahary et al. 2002). According to Grinnell et al. (1994) research performed in the 1950's implicated "connective tissue cells" in wound contraction and that subsequently, Gabbiani in 1972 demonstrated that granulation tissue could undergo a "smooth muscle-like" contraction *in vitro*. The cells in this granulation tissue exhibited some features of smooth muscle cells such as actin filament bundles or stress fibres. These 'myofibroblasts' were therefore proposed to be responsible for force generation, and their presence has turned out to be a general feature of tissues undergoing contraction (Grinnell 1994). In a normally healing wound when contraction stops and the wound is fully epithelialised myofibroblasts disappear in the scar. This could be due to myofibroblasts becoming quiescent when the wound is closed or myofibroblasts disappearing through apoptosis (Desmouliere 1995). It has been noted that hypertrophic scar tissue, but not keloid tissue, contains elevated numbers of myofibroblasts (Scott, Ghahary et al. 2002; Desmoulière, Chaponnier et al. 2005). Therefore, the presence of myofibroblasts has been considered a marker for fibrous tissue that is prone to undergo contracture. Keratinocytes have also been implicated in contracture formation. Work in the MacNeil group at the University of Sheffield has shown that keratinocytes are the primary cell type responsible for contraction of tissue engineered skin (Ralston, Layton et al. 1999; Chakrabarty, Heaton et al. 2001; Harrison, Gossiel et al. 2006c; Thornton, Harrison et al. Provisionally accepted; Harrison and MacNeil Submitted for publication). Erdag and Sheridan (2004) showed that the addition of fibroblasts to a cultured skin substitute prepared from acellular dermis seeded with keratinocytes reduced contraction, increased vascularisation and produced a better epidermis (Erdag and Sheridan 2004). However the addition of keratinocytes to fibroblast populated collagen gels increased contraction (Oshita, Lee et al. 2006). Souren et al. (1989) showed that keratinocyte mediated contraction of collagen gels was more effective when the cells were seeded

on top of the gel rather than when they were dispersed through it. It has also been shown that inhibition of keratinocyte differentiation inhibits reconstructed skin contraction (Thornton, Harrison et al. Provisionally accepted). This correlates with Lillie et al. (1988) who suggested that contraction occurs in the stratum spinosum. The ability of keratinocytes to contract collagen gels or dermis is closely related to the concentration of collagen in the gel (Lillie, MacCallum et al. 1988) or the pliability of the dermis (Chakrabarty, Heaton et al. 2001; Harrison, Gossiel et al. 2006c). It has been shown that contraction ultimately becomes irreversible and appears to be maintained by collagen crosslinking (Harrison, Gossiel et al. 2006c). In a recent review Harrison and MacNeil state that it is clear that despite 50 years of research in this area, the treatment and prevention of graft contraction have progressed very little and understanding of the underlying mechanism remains poor (Harrison and MacNeil Submitted for publication).

Most likely, the process of contraction evolved as a rapid and efficient way to close an open wound (Greenhalgh 2002). Loose-skinned animals possess a subcutaneous muscle layer called the panniculus carnosus which enables the skin to glide smoothly over the underlying tissues leading to wound closure with little scaring or loss of function. However, in man, this muscular layer is absent and the skin is more firmly attached, so the consequences of wound contraction are less beneficial, ranging from a minimal cosmetic scar in some cases to deformity or disability in others (Germain, Jean et al. 1994; Grinnell 1994; Harrison and MacNeil Submitted for publication). It is therefore necessary to distinguish between contraction as a normal, integral part of the wound healing process and excessive contracture the abnormal result of the wound healing process resulting in significant scaring, disfigurement or loss of mobility. Scar contractures occur when the scar is not fully matured and often tend to be hypertrophic. They are common after burn injury across joints or skin concavities (Bayat, McGrouther et al. 2003).

1.2.5 Pharmacological methods to reduce contraction – β-aminopropionitrile and 4-methyl umbelliferone.

β-aminopropionitrile (βAPN) is a specific non competitive lysyl oxidase inhibitor isolated from the sweet pea (Arem, Madden et al. 1975). Lysyl oxidase enzymes are

a family of enzymes involved in the crosslinking of collagen. It has been shown that fibroblasts treated with 0.25 and 0.5 mM βAPN have reduced migration however no alteration in cell proliferation or collagen synthesis was observed (Nelson, Diegelmann et al. 1988). Harrison et al. (2006a) showed that treatment of keratinocyte and fibroblast mono and co-cultures with βAPN increased production of P1NP the amino terminated procollagen tripeptide. This drug was subsequently shown to decrease the contraction of the MacNeil group tissue engineered skin model from 60% of original area to approximately 20% of original area at a concentration of 200 μg.ml^{-1} in Greens medium (Harrison, Gossiel et al. 2006c). This is in good agreement with research which showed that treatment of fibroblast populated collagen gels with βAPN at a concentration of 1 mM decreased contraction (Redden and Doolin 2003).

Chakrabarty (2001) and Ralston (1997) showed that contraction of the reconstructed skin model was primarily driven by keratinocytes. It has also been shown that the more differentiated the keratinocytes were the more the skin contracted (Thornton, Harrison et al. Provisionally accepted). Reducing the hyaluronan concentration around the keratinocytes, temporarily reducing the differentiation of the keratinocytes could therefore reduce contraction. Rilla et al. (2005) reported that excessive epidermal HA was implicated in the hyperproliferation and disturbed terminal differentiation of keratinocytes. 4-methyl umbelliferone (4-MU) inhibits the synthesis of hyaluronan by suppressing the expression of hyaluronan synthase (HAS) enzymes on the plasma membrane of keratinocytes (Rilla et al. 2005). Rilla et al. 2005 also showed that decreasing HA synthesis by treating keratinocyte cultures with 4-MU strongly inhibited cell proliferation. In monolayer cultures treated with 0.5 mM 4-MU cell proliferation was completely, but reversibly, blocked. In a more physiologically relevant model keratinocytes were grown on a collagen gel at an air-liquid interface to create a fully differentiated normal epidermis. 4-MU did not affect the structure or the differentiation pattern of the cultures (Rilla, Pasonen-Seppanen et al. 2004). 4-MU has also been shown to increase mRNA expression for MMP-1 (Interstitial collagenase) resulting in activation of MMP-2 (Gelatinase A / Type IV collagenase) in cultured human skin fibroblasts (Nakamura, Ishikawa et al. 2002).

Hyaluronan (HA) is a negatively charged extracellular glycosaminoglycan (GAG) that accumulates in wound beds. Allison and Grande-Allen (2006) reviewed the uses of HA in tissue engineering where it has been used in applications as far ranging as tissue engineering scaffolds and drug delivery devices. This review indicates that HA is crucial in embryo development, tissue organisation, wound healing and angiogenesis. It is most widely known, however for its effects on the biomechanical properties of tissues especially in cartilage. The presence of HA in wound beds increases keratinocyte migration (Karvinen, Pasonen-Seppanen et al. 2003; Allison and Grande-Allen 2006). HA also has an important role in cell adhesion, migration, proliferation and differentiation (Nagira, Nagahata-Ishiguro et al. 2007). Nagira et al. (2007) investigated how sulphated HA (sHA) influenced the adhesion of fibroblasts and keratinocytes. Nagira et al. also investigated how sHA affected keratinocyte proliferation. Differentiation was investigated by analysis of Wnt4, Wnt5a, Wnt6, Wnt7a, Notch 1, Notch2 and Notch3 expression. This study showed that sHA promoted keratinocyte differentiation by increasing the expression of Wnt4 and Wnt6 mRNA's. Conversely Notch1 expression was down regulated whilst Notch2 expression remained unchanged and Notch3 expression increased. Fibroblasts showed low adhesion to sHA coated surfaced whilst keratinocytes showed high adhesiveness to the same coated surfaces. HA interacts with cell surfaces via cell-surface receptors such as CD44 (Allison and Grande-Allen 2006). It was stated in this paper that the interaction of HA with CD44 promotes cell migration, extracellular matrix remodelling, promotes the inflammatory response and can inhibit cell adhesion.

Reducing the hyaluronan concentration around the keratinocytes, by treatment with 4-MU, might temporarily reduce the differentiation of the keratinocytes and reduce contraction in the reconstructed skin model.

1.2.6 Burns wounds.

Burn trauma to the skin can occur through a variety of methods, flames, scalds, contact, electrical, chemical, friction and sunburn (Benson, Dickson et al. 2006). Burn depth is traditionally divided into four categories; first degree burns affect the epidermis only. Second degree burns extend into the dermis whilst third degree

burns extend into the subdermal fat. Fourth degree burns extend beyond the subdermal fat into the fascia, bone, tendon, muscle or other tissue, this is illustrated in figure 1.3 (Greenhalgh 2002).
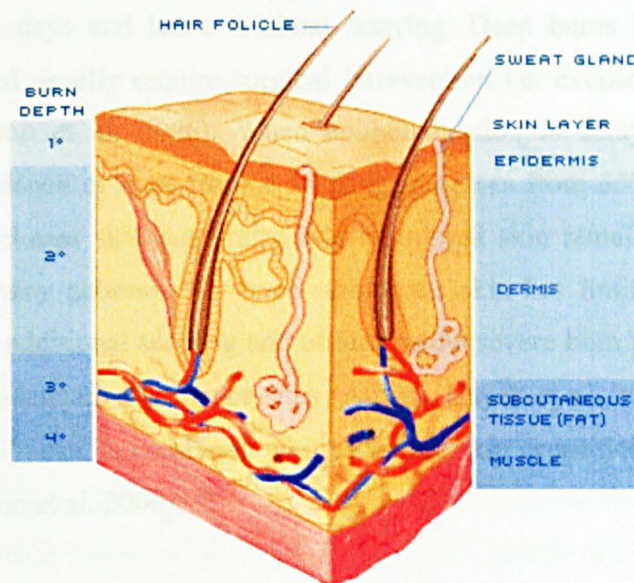
Figure 1.3 Illustration of burn depth in relation to the gross morphology of skin. Image obtained from http://www.burn-recovery.org/treatment.htm.

Burn injuries can also be divided into three zones, the zone of coagulation, zone of stasis and zone of hyperaemia (Quinn, Courtney et al. 1985). The zone of coagulation is the most severely affected part of the burn. Here there is irreversible tissue loss due to the coagulation of the ECM proteins. The zone of stasis surrounds the zone of coagulation. Here the tissue is potentially salvageable, however this zone is characterised by decreased tissue perfusion. The zone of hyperaemia is the outermost zone and here tissue perfusion is increased. The tissue here will usually recover (Hettiaratchy and Dziewulski 2004b). Burns are peculiar injuries because they force an organism into a tremendous biological task of equilibrating its deranged internal homeostasis and, at the same time, reconstructing its destroyed protective barrier against the external environment (Linares 2002). However the burn wound is a dynamic living environment that will alter depending on both intrinsic factors (inflammation, bacterial contamination etc.) and extrinsic factors (dehydration, systemic hypotension, cooling etc.) (Papini 2004).

Healing of burn wounds follows the same principles as general wound healing. The type of healing that is involved changes, depending on the depth of the wound (Greenhalgh 2002). Superficial burns usually heal primarily by reepithelialisation within fourteen days and leave minimal scarring. Deep burns have a prolonged healing time and usually require surgical intervention i.e. excision and skin grafts (Benson, Dickson et al. 2006). When epithelialisation is delayed beyond three weeks, the incidence of hypertrophic scarring increases from 33% to 78% (Papini 2004). Split-thickness skin autografts from uninjured skin remain the mainstay of treatment for many patients. However autologous skin has limited availability, is associated with additional scarring and unfortunately severe burn patients invariably lack sufficient adequate skin donor sites (Atiyeh, Hayek et al. 2005). If this is the case then STSG's can be combined with cadaveric skin or artificial skin substitutes (Benson, Dickson et al. 2006).

According to Bombaro et al. (2003) hypertrophic scarring following burn injury continues to be a real concern and clinical challenge. It is common for burn survivors to wear pressure garments in an attempt to reduce hypertrophic scarring. When reviewing the prevalence of hypertrophic scaring following burns Bombaro et al. (2003) noted that 67% of the group examined presented with hypertrophic scars. The group was split by race into white versus non-white and the results are shown in table 1.1 below.

Table 1.1 Prevalence of hypertrophic scarring after burns taken from (Bombaro, Engrav et al. 2003)

Age / Years	White (%)	Non-white (%)
<15 (n=13)	6/8 (75%)	5/5 (100%)
15-44 (n=48)	19/32 (60%)	12/16 (75%)
45-65 (n=23)	13/19 (68%)	3/4 (75%)
>65 (n=2)	0/2 (0%)	-

These results correlate with studies performed by Deitch et al. and McDonald and Deitch (Deitch, Wheelahan et al. 1983; McDonald and Deitch 1987) who showed that non-white patients were more likely to have hypertrophic scaring after burns (Deitch, Wheelahan et al. 1983) or in burns that had been treated with a skin graft

(McDonald and Deitch 1987). Children, under 14 years of age, were also more likely to have hypertrophic scars after burns than adults especially in grafted sites (McDonald and Deitch 1987). As mentioned previously the current gold standard for burns wound treatment is the addition of an autologous split thickness skin graft (STSG) to the wound bed. However treatment with STSG's is not problem free, STSG's have been shown to produce hypertrophic scars and contractures (McDonald and Deitch 1987). Stephenson et al. (2000) therefore investigated the effect of full thickness skin grafts (FTSG's) on contraction. Unfortunately these grafts still contracted significantly despite the initial thought that FTSG's should contract less. However there was no comparison made between FTSG's and STSG's in this study.

Currently problematic scars can be treated with non-invasive treatment, invasive treatment or be left alone and managed. Non-invasive treatments include pressure therapy i.e. pressure garments, splitting, acrylic casts, masks and clips, various oils, drugs, creams and lotions. Hydrotherapy and massage therapy are also used. Invasive treatments can involve surgical excision and revision, steroid injections, fluorouracil injections, interferon-γ, bleomycin, radiotherapy, laser therapy and cryosurgery (Bayat, McGrouther et al. 2003). Pressure garments have been the major form of treatment for hypertrophic scars and potential contractures since the early 1970's. Specific references to treating hypertrophic scars with pressure go as far back as the early 1800's (Edgar and Brereton 2004; Macintyre and Baird 2006). Macintyre et al. (2006) when reviewing pressure garments suggest that the pressure exerted by pressure garments controls collagen synthesis, reduces collagen production and encourages realignment of collagen bundles already present. They suggest that these effects could increase scar maturation, reduce the incidence of contractures and thus the need for surgical intervention. A reduction in the itchiness and pain that is associated with active hypertrophic scars is a fortunate side effect of the application of pressure to scars (Macintyre and Baird 2006). However, although pressure therapy has been used for more than three decades in the management of hypertrophic and burn scars, so far scientific evidence is limited. Recommendations about the amount of pressure and duration of the therapy are merely based on empirical observations, although, there is consensus that the early application of pressure is necessary for an optimal outcome (Williams, Knapp et al. 1998; Van Den Kerckhove, Stappaerts et

al. 2005). Van Den Kerckhove et al. did show a significant difference in the thickness of post-burn scars that were preventively treated with garments that were delivering pressures with a mean value of 15 mmHg compared to scars treated with garments delivering a lower mean pressure (Van Den Kerckhove, Stappaerts et al. 2005).

1.3 Wound healing and contraction models.

Several different contraction models are available for use; they vary from simple collagen gel models to *in vivo* animal models. All of these models have positives and negatives. The collagen gel model is very popular especially when populated with fibroblasts. It is relatively easy to cast collagen gels into the required shapes for mechanical loading (Berry, Shelton et al. 2003), for clamping to allow cellular contractile forces to be measured (Freyman, Yannas et al. 2002) or for clamping for microscopy (Freyman, Yannas et al. 2001). Collagen gels contract rapidly and experiments have been performed over time periods varying from 3 hours (Deveci, Gilmont et al. 2005) to 28 days (Helary, Giraud Guille et al. 2005; Helary, Ovtracht et al. 2006). Models based on dermal equivalents or reconstructed skin are also used and are especially popular in the MacNeil group at the University of Sheffield. In this group a model based on sterilised acellular human dermis has been used since the mid 1990's. Its uses vary from investigating skin graft contraction (Ralston, Layton et al. 1997; Chakrabarty, Heaton et al. 2001; Harrison, Gossiel et al. 2006c; Thornton, Harrison et al. Provisionally accepted) and other wound healing processes (Dawson, Goberdhan et al. 1996; Harrison, Heaton et al. 2006b) to investigating melanoma invasion through the basement membrane (Eves, Layton et al. 2000; Eves, Katerinaki et al. 2003) and studies of skin pigmentation. The uses of this model have recently been reviewed by MacNeil (2007). Animal models could also be used although there are complications associated with the presence of the panniculus carnosus allowing for the free movement of the skin over the underlying tissue.

1.3.1 Collagen gels.

Several *in vitro* models of wound contraction have been developed using fibroblasts cultured in collagen or fibrin matrices (Grinnell 1994). The majority of research into

contraction has been performed using fibroblast populated collagen gels. The fibroblast populated collagen gel model was introduced by Bell and Ivarsson in 1979 and has since been widely used for investigating contraction, (Bell, Ivarsson et al. 1979; Bellows, Melcher et al. 1982; Schaffer, Tantry et al. 1997; Enever, Shreiber et al. 2002; Howling, Dettmar et al. 2002; Berry, Shelton et al. 2003; Gentleman, Nauman et al. 2004). However collagen matrix contraction requires serum and can be stimulated by individual extracellular factors, including $TGF_{\beta 1}$ and PDGF or inhibited by factors such as epidermal growth factor (EGF) (Schaffer, Tantry et al. 1997). When fibroblasts are cultured within an extracellular matrix such as collagen or fibrin, fibroblasts experience a richer, more complex physical environment and markedly different geometry which is more physiologically relevant than cells on 2D surfaces (Bellows, Melcher et al. 1982; Grinnell 2003).

According to Grinnell (2003), Paul Weiss showed that fibroblasts cultured in a blood plasma clot varied in shape from stellate to bipolar depending on the orientation of the fibrous network of the clot. In free floating collagen gels where there is little resistance to cellular force fibroblasts can project an elaborate dendritic network of extensions interconnected by gap junctions. If the matrix is constrained then tension develops within the matrix and the collagen fibrils become orientated in response to the force exerted by the cells (Grinnell 2003). It is thought that changes in cell proliferation and biosynthetic activity after stress relaxation provide insight into the possible mechanism of myofibroblast disappearance at the end of wound healing. As long as the tissue is under mechanical stress, cell proliferation and biosynthetic activity will persist. Once mechanical stress is relieved, usually by a combination of wound contraction and biosynthetic activity, cells will switch to a non-proliferative phenotype. Thus fibroblasts in floating gels and those in anchored collagen gels show profound differences in cell proliferation (Grinnell 1994).

Germain et al. (1994) used collagen gels to compare the contractile properties of fibroblasts obtained from samples of granulation tissue and dermal fibroblasts. It was noted that collagen gels prepared with dermal fibroblasts contracted by 25% of their original area over the first 24 hours whilst the gels seeded with wound healing fibroblasts contracted by 50% of their original area over the first 24 hours. However

after fourteen days in culture there was no significant difference in the surface area of the two gels (Germain, Jean et al. 1994). Schäffer et al. (1997) and Moulin et al. (1998) also showed that wound healing myofibroblasts contract collagen gels more than dermal fibroblasts. Wound fluid also increased the contraction of collagen gels populated with dermal fibroblasts but had no effect on the contraction of collagen gels populated with wound healing fibroblasts (Schaffer, Tantry et al. 1997). The concentration of collagen in the gel can also affect fibroblast behaviour with the rate of gel contraction varying inversely with the collagen concentration (Bell, Ivarsson et al. 1979). Helary et al. prepared collagen gels with collagen concentrations of 5 mg.ml^{-1} and 40 mg.ml^{-1}. The gels with low collagen concentration showed weak myofibroblast proliferation and no apoptosis, however the cells migrated easily. In gels with a high collagen concentration, cell proliferation and apoptosis occurred and the cells spread more than in the loose matrices (Helary, Ovtracht et al. 2006). Expression of α smooth muscle actin (αSMA) and MMP1 (collagenase 1) did not differ between the 5 mg.ml^{-1} and 40 mg.ml^{-1} matrices. However, expression of MMP2 (gelatinase A) was increased in the high density (40 mg.ml^{-1}) matrices (Helary, Giraud Guille et al. 2005). Pullar and Isseroff (2005) used fibroblast populated collagen gels to investigate the role of the adrenergic signalling system in contraction. Treatment of the gels with a β-agonist delayed contraction in a concentration dependent manner whilst treatment with a β2-adrenergic receptor specific antagonist reduced the delay in contraction. The authors report that the β2-adrenergic receptor is therefore solely responsible for the delay in contraction and further showed that this is via a cyclic adenosine monophosphate (cAMP) mechanism (Pullar and Isseroff 2005).

Shannon et al. (2006) used collagen gels to compare buccal mucosa and dermal fibroblasts. Collagen gel contraction, αSMA expression, growth factor production and the effects of TGF-β$_{1\&3}$ were investigated. In this study oral fibroblasts contracted collagen gels significantly more than the dermal fibroblasts. The presence of TGF-β$_1$ significantly increased collagen gel contraction in a concentration dependent manner in both cultures. TGF-β$_3$ significantly increased collagen gel contraction in both cultures. In collagen gels populated with oral fibroblasts this was in a concentration dependent manner but this was not the case in collagen gels

populated with dermal fibroblasts (Shannon, McKeown et al. 2006). TGF-β has also been shown to increase contraction of fibroblast populated collagen gels in a concentration dependent manner by Grinnell and Ho (2002). The addition of keratinocytes into fibroblast populated collagen gels also leads to an increase in contraction (Oshita, Lee et al. 2006).

Collagen gel models are especially popular for investigating the effects of various drugs on fibroblast biology and contraction. Redden and Doolin produced fibroblast populated collagen gels containing both a high and a low concentration of fibroblasts. These gels were treated with the lathyrogen β-aminopropionitrile (βAPN) fumarate. The βAPN reduced the contraction of both of the collagen gels (Redden and Doolin 2003). Howling et al. investigated the effect of adding chitosan, chitin and hyaluronan to the culture medium surrounding the fibroblast populated collagen gels. Adding chitosan to the medium inhibited contraction whilst chitin and hyaluronan had no significant effect (Howling, Dettmar et al. 2002). Moulin et al. (1998) treated collagen gels populated with both wound healing and dermal fibroblasts with TGF-β_1 and interferon-γ (IFNγ). Treating both collagen gel populations with TGF-β_1 initially increased contraction in both cell populations but this increase was stronger with the wound healing fibroblasts. The effect of treatment with IFNγ was the same for both fibroblast populations. Contraction was reduced during the initial 24 hours and then stopped completely thereafter, this also proved to be reversible as once the IFNγ was removed from the culture system after a 2 day delay contraction resumed in the dermal fibroblast populated gels. In wound healing fibroblast populated gels very little, if any, contraction was observed when the IFNγ was removed (Moulin, Castilloux et al. 1998).

Whilst the collagen gel model is simpler, cheaper and less time consuming collagen gels are limited by their lack of dermal architecture and basement membrane. A number of factors can influence the effectiveness of this assay. The concentration of the collagen in the gel, being a major factor. Personal experience also illustrated that the age of the cells particularly the age of the keratinocytes had implications on the success of the experiment. Keratinocytes that had been subjected to more than two passages struggled to attach to the gels and therefore these gels did not contract as

much as gels seeded with lower passage number keratinocytes. However these factors do not alter the fact that this model is the accepted model for investigating contraction. The time taken to run the experiments, six or seven days, is much shorter than the time taken to run other contraction model experiments i.e. reconstructed skin models. This makes collagen gels a useful screening tool to quickly investigate the effects of a range of drugs at a wide range of concentrations on contraction. The addition of keratinocytes to the standard fibroblast populated collagen gels makes the model more of a reconstructed skin model but the absence of a basement membrane and dermal architecture cannot be overcome and this should be taken into consideration when reviewing the data.

1.3.2 Tissue engineered skin equivalents.

Skin equivalents for contraction studies have been produced using a variety of different methods. Most of the skin or dermal equivalents are produced using acellular dermis and primary skin cells, dermal fibroblasts and keratinocytes. It is possible to produce acellular dermal matrices in a variety of methods. Erdag and Sheridan (2004) report the production of an acellular dermal matrix by rapidly freeze thawing cadaver skin. These matrices were seeded with fibroblasts and / or keratinocytes and transplanted onto mice. The presence of fibroblasts in these matrices reportedly produced a thicker epidermis, reduced contraction and enhanced angiogenesis compared to matrices seeded with keratinocytes alone (Erdag and Sheridan 2004). Dawson et al. (1996) and Ralston et al. (1997) report the production of a sterilised deepithelialised acellular dermis (DED) using increasing concentrations of glycerol. Ralston et al. (1997) used this composite to illustrate that whilst fibroblasts can contract this composite slightly, when keratinocytes are added, composite contraction is more pronounced. Ghosh et al. (1997) investigated how changing sterilisation methods affected the quality of the resulting skin composites. Glycerol sterilisation and lyophilisation were used to render the skin acellular. Ethylene oxide treatment and gamma irradiation were used to sterilise the skin. Deepidermisation was carried out by immersion of the skin in 1 M sodium chloride solution, phosphate buffered saline (PBS) or dispase solution. It was noted in this study that skin can be reconstructed based on either glycerol or ethylene oxide treatment but the ethylene oxide treatment produced composites with poorer

epidermis' than the glycerol treated composites (Ghosh, Boyce et al. 1997). This model has subsequently been used to produce epidermal-dermal composites for clinical use and to investigate the mechanism of skin graft contraction especially the role of the keratinocyte (Chakrabarty, Dawson et al. 1999; Chakrabarty, Heaton et al. 2001; Harrison, Heaton et al. 2006b; Harrison, Gossiel et al. 2006c; Thornton, Harrison et al. Provisionally accepted). Harrison et al. used this model to investigate pharmacological approaches to reduce contraction. Agents such as βAPN, α2-macroglobulin and catechin reduced contraction whilst estrone increased contraction (Harrison, Gossiel et al. 2006c). This model was also used by Eves et al. to investigate melanoma invasion in skin (Eves, Layton et al. 2000; Eves, Katerinaki et al. 2003). It has been shown that contraction of this skin composite can be affected by a number of factors summarised in table 1.2

Table 1.2 Effect of various agents on the contraction of reconstructed skin.

Agent	Effect on contraction	Reference
Ascorbic acid-2-phosphate	Increased	(Chakrabarty, Heaton et al. 2001)
Mannose-6-phosphate	No significant effect	(Chakrabarty, Heaton et al. 2001)
Forskolin	No significant effect	(Chakrabarty, Heaton et al. 2001)
Galardin	Decreased	(Chakrabarty, Heaton et al. 2001)
Keratinocyte conditioned medium	Decreased	(Chakrabarty, Heaton et al. 2001)
Glutaraldehyde pre-treatment	Decreased	(Harrison, Gossiel et al. 2006c)
Insulin like growth factor	No significant effect	(Harrison, Gossiel et al. 2006c)
Estrone or Estradiol	Increased	(Harrison, Gossiel et al. 2006c)
Dexamethasone	No significant effect	(Harrison, Gossiel et al. 2006c)

Basic fibroblast growth factor	No significant effect	(Harrison, Gossiel et al. 2006c)
Tumour necrosis factor α	No significant effect	(Harrison, Gossiel et al. 2006c)
Prostaglandin-E_2	No significant effect	(Harrison, Gossiel et al. 2006c)
Catechin	Decreased	(Harrison, Gossiel et al. 2006c)
α_2-macroglobulin	Decreased	(Harrison, Gossiel et al. 2006c)
β-aminopropionitrile	Decreased	(Harrison, Gossiel et al. 2006c)
Putrescine	No significant effect	(Harrison, Gossiel et al. 2006c)
NTU283 or NTU285*	No significant effect	(Harrison, Gossiel et al. 2006c)

*NTU283 and NTU285 are novel thioimidazolium transglutaminase inhibitors synthesised by Prof. M. Griffin at Nottingham Trent University.

Ascorbic acid-2-phosphate is a stable form of vitamin C and stimulates procollagen synthesis. It is a cofactor for hydroxyproline and hydroxylysine (Chakrabarty, Heaton et al. 2001). Mannose-6-phosphate inhibits the effects of TGF-β (Chakrabarty, Heaton et al. 2001). Forskolin stimulates adenylate cyclase and elevates cAMP (Chakrabarty, Heaton et al. 2001). Galardin inhibits matrixmetalloproteinases (Chakrabarty, Heaton et al. 2001). Glutaraldehyde pre-treatment reduces dermal pliability (Harrison, Gossiel et al. 2006c). Insulin like growth factor, Estrone and Estradiol stimulate collagen synthesis (Harrison, Gossiel et al. 2006c). Dexamethasone and basic fibroblast growth factor inhibit collagen synthesis (Harrison, Gossiel et al. 2006c). TNF-α also inhibits collagen synthesis it also inhibits keratinocyte proliferation (Harrison, Gossiel et al. 2006c). Prostaglandin-E_2 inhibits collagen synthesis and stimulates keratinocytes proliferation (Harrison, Gossiel et al. 2006c). Catechin, an antioxidant, inhibits collagen degradation (Harrison, Gossiel et al. 2006c). α_2-macroglobulin also inhibits

collagen degradation and inhibits MMP's (Harrison, Gossiel et al. 2006c). βAPN is a non-competitive lysyl oxidase inhibitor and inhibits collagen crosslinking (Harrison, Gossiel et al. 2006c). Putrescine is a competitive transglutaminase inhibitor, inhibiting collagen crosslinking (Harrison, Gossiel et al. 2006c). NTU283 is an irreversible transglutaminase inhibitor, NTU285, a competitive transglutaminase inhibitor (Harrison, Gossiel et al. 2006c).

Investigation into the mechanism by which the reconstructed skin model contracts showed that collagen synthesis and degradation (Harrison, Gossiel et al. 2006c) had no effect on contraction whilst collagen crosslinking (Harrison, Gossiel et al. 2006c) and keratinocyte differentiation (Thornton, Harrison et al. Provisionally accepted) significantly affected contraction. Blocking the action of lysyl oxidase by the addition of βAPN to the composite culture medium significantly reduced contraction at βAPN concentration of 200 $\mu g.ml^{-1}$ (Harrison, Gossiel et al. 2006c).

Tissue engineered skin equivalents, especially those based on DED have three main advantages over collagen gels especially fibroblast populated collagen gels. These are namely the presence of basement membrane proteins, an appropriate dermal architecture and the presence of multiple cell types. The main disadvantage to this model is the lack of suitable skin that can be made into DED. Whilst this laboratory is lucky in the quantity of skin that it receives the vast majority of the skin is only suitable for cell isolation. Skin of sufficient quality and quantity for DED production is rarely obtained. These experiments also take a long time to run i.e. 28 days instead of the 6 or 7 days taken for collagen gel contraction experiments. However this contraction model is a useful model to verify the results obtained from the collagen gel experiments. Once the collagen gels have been used to identify drugs that could be used to block or reduce contraction and at what concentrations.

1.3.3 Animal models.

As mentioned in section 1.3 the presence of the panniculus carnosus in animals makes the majority of animals unsuitable for contraction studies. Selection of an animal model depends on a number of factors including availability, cost, ease of handling, investigator familiarity, and anatomical/functional similarity to humans

(Sullivan, Eaglstein et al. 2001). According to Middelkoop et al. (2004) small mammals, such as mice, rats, rabbits and guinea pigs are suitable for studies that require large numbers of animals or specific characteristics, such as availability of knockouts or transgenic animals (mainly available in mice) (Escamez, Garcia et al. 2004) or a compromised immune system (athymic mice). Wound healing in rodents differs considerably from wound healing in humans as wounds in rodents heal primarily by contraction rather than migration of epidermal cells (Middelkoop, Van Den Bogaerdt et al. 2004). Sullivan et al. evaluated a number of different wound healing models and compared them to wound healing in humans. The porcine model agreed with human studies 78% of the time whilst small mammals agreed with human studies 53% of the time and *in vitro* models 57% of the time (Sullivan, Eaglstein et al. 2001). Sullivan et al. do state that because no model will completely replicate clinical human wound healing, it is essential that the model utilized be selected with care. For example guinea pigs are generally used to evaluate the effects of vitamin C deficiency on wound healing as guinea pigs, like humans, require vitamin C from dietary sources. Comparatively few other animals, such as pigs, synthesise their own vitamin C but the anatomy and physiology of pig skin is similar to human skin and neither mammal possesses a panniculus carnosus (Sullivan, Eaglstein et al. 2001). This makes the porcine model more suitable for the evaluation of therapeutic agents that could, for example, be used to treat scarring. Middelkoop et al. (2004) report the use of a porcine burn model to evaluate the efficacy of various treatments whilst looking at reepithelialisation, inflammatory response, contraction and bacterial contamination. Horan et al. (2005) used mice to evaluate the effect of stress on wound contraction. The mice were restrained and deprived of food and water for set periods over the experiment. These mice showed delayed healing and reduced contraction compared to those mice just deprived of food and water for the same time periods but not restrained. Mirastschijski et al. (2004) used Sprague-Dawley rats to evaluate the effect of a broad spectrum MMP inhibitor on wound healing. The MMP inhibitor reduced wound contraction, decreased myofibroblast formation but impaired keratinocyte migration but not keratinocyte proliferation, and produced abnormal granulation tissue and blood vessel architecture. Spyrou and Naylor (2002) used Hooded Lister rats to investigate the effect of basic FGF (FGF_2) on scarring. FGF_2 was shown to inhibit the

differentiation of fibroblasts into myofibroblasts and improved the dermal architecture of the healing wound.

Despite the limitations imposed by the differences in the anatomy and physiology of animal skin compared to human skin these animal models are still used to evaluate wound treatments. For the purpose of this project an animal model would not be required. However, if this wound dressing were to be taken forward to the clinic an animal model, ideally a porcine model, would be needed.

1.4 Wound dressings and skin replacements.

Mankind has been covering wounds with a variety of materials since the earliest written records. Historically, wounds have been treated with homemade remedies derived from ritualistic teachings and observation. According to Lionelli and Lawrence the "three healing gestures" were described c2200 BC on an ancient clay tablet: 1. washing the wound, 2. making plasters (mixtures of herbs, ointments, and oils that were applied to wounds to aid in the healing process), and 3. bandaging the wound. References to early wound care are seen in the Bible, ancient Assyrian writings and ancient Greek texts such as The Iliad and The Odyssey by Homer and writings by Hippocrates in 400 BC (Ramos-E-Silva and Ribeiro De Castro 2002; Lionelli and Lawrence 2003) According to Ovington (2007) Sumerian cuneiform tablets from before 2000 BC describe the application of poultices formed of mud, milk and plants to wounds. Egyptian papyruses from 1550 and 1650 BC provide specific details of how to wash the wound, prepare and apply plasters of honey, plant fibres, and animal fat and then bandage the wound (Ovington 2007). In the Ebers and Edwin Smith papyri (1500-1600 BC) there are descriptions of a variety of dressing materials used in ancient Egypt, including bandages with grease which can be seen as precursors of tulle gras, one of the earliest non-adherent dressings, which was used during the First World War (Lawrence 1982; Lionelli and Lawrence 2003). Prior to the early 1960's however dressings had hardly advanced from those used in ancient times with dressings consisting primarily of dry non-occlusive gauze and non-woven cotton or wool (Cho and Lo 1998).

Immediate wound coverage is one of the cornerstones of wound management. Acute or chronic wounds can usually be covered by any of a number of synthetic and natural dressings (Singer and Clark 1999). However different sized wounds require different strategies for closure. The goal should always be to obtain the most cosmetic and functional wound closure the first time. Whilst small and clean wounds such as a small burn wound or scar can be treated with excision and sutures or staples resulting in a relatively narrow scar this is dependent on the size of the wound. If infection is suspected the wound can be left open and treated with dressing changes for 4-5 days and then closed (Greenhalgh 2002). It is not possible to treat larger wounds like this and so for larger areas of skin loss, skin grafts are usually used either as sheets or expanded by meshing. According to Greenhalgh (2002) there are three stages of graft healing, the first being the phase of imbibition, in this phase the graft 'imbibes' or survives by diffusion of nutrients from the wound bed. During this phase which lasts approximately 2-3 days if there is any barrier to the diffusion of nutrients to the graft such as a blood clot, oedema, or nonviable tissue the graft will not 'take' and will therefore be lost. The second phase of graft healing is the phase of neovascularisation where new blood vessels invade the graft through angiogenesis and 'hook up' with those in the graft. The final stage is the phase of maturation where collagen bridges form between the graft and the wound bed. This phase closely mirrors the maturation phased in wound healing described in section 1.2.3 (Greenhalgh 2002). The ideal biologic skin substitute or wound dressing has to meet a formidable list of criteria listed below which have been taken from Ehrenreich and Ruszczak (2006) and added to from Seal et al. (2001), Sheridan and Tompkins (2002) and Atiyeh et al. (2005):

- Absence of antigenicity
- Tissue compatible
- Absence of local or systemic toxicity
- Impermeable to exogenous microorganisms
- Water vapour transmission similar to normal skin
- Rapid and sustained adherence to the wound surface
- Conform to surface irregularities
- Elastic to permit motion of underlying tissue

- Tensile strength to resist fragmentation

- Inhibition of wound surface flora and bacteria

- Long shelf life, minimal storage requirements

- Biodegradable (for permanent membranes)

- Low cost

- Minimise nursing care of wounds

- Minimise patient discomfort

- Translucent properties to allow direct observation of healing (for dressings)

- Reduce healing time

- Not increase rate of infection

- Patient acceptance

- Grow with a child

- Can be applied in one operation

- Prevent heat and fluid loss

- Does not become hypertrophic or contract

Currently there are no materials commercially available that meet all these criteria. However commercial products, such as Permaderm, are beginning to emerge to meet this need (MacNeil 2007), and whilst autologous skin grafts, especially STSG's are the current gold standard for treatment of large burns there are a number of problems associated with them. Among these are donor site morbidity and the lack of available skin especially in patients with extensive severe burns. In essence when skin for an autologous graft is taken the surgeon is increasing the total body surface area affected by the burn, therefore potentially increasing the problems associated with large wounds i.e. fluid loss, and increasing the chance of infection. It has therefore been necessary to look at various different methods for closing wounds that could either be used in the place of, or in conjunction with STSG's. Table 1.3 summarises some commercially available skin substitutes.

Table 1.3 Table summarising the materials from which some commercially available epidermal, dermal and skin replacements are made.

Trade name	Materials used	Cells included	Reference
Alloderm (Dermal substitute)	Acellular deepithelialised cadaver dermis	-	(Jones, Currie et al. 2002)

Apligraf (Skin replacement)	Collagen	Neonatal allogenic fibroblasts & keratinocytes	(Jones, Currie et al. 2002)
Biobrane (Temporary skin substitute)	Silicone, nylon mesh, collagen	-	(Jones, Currie et al. 2002)
BioSeed-S (Epidermal substitute)	Fibrin glue	Cultured autologous keratinocytes	(Enoch, Grey et al. 2006b)
CellSpray (Epidermal substitute)	-	Cultured autologous keratinocytes	(Enoch, Grey et al. 2006b)
Dermagraft (Dermal substitute)	Polyglycolic acid or polyglactin-910	Neonatal allogenic fibroblasts	(Jones, Currie et al. 2002)
Epicel (Epidermal replacement)	-	Cultured autologous keratinocytes	(Jones, Currie et al. 2002)
E-Z-Derm (Temporary skin substitute)	Chemically crosslinked porcine collagen	-	(Ramos-E-Silva and Ribeiro De Castro 2002)
Human Amniotic membrane (Dermal substitute)	Allogenic human amnion	-	(Atiyeh, Hayek et al. 2005)
Hyaff-NW (Dermal substitute)	Benzyl-esterified hyaluronan derivative	Autologous fibroblasts	(Atiyeh, Hayek et al. 2005)
Integra (Dermal Substitute)	Silicone, collagen, glycosaminoglycans	-	(Jones, Currie et al. 2002)
Laserskin (Epithelial cover)	Hyaluronic acid	Cultured autologous keratinocytes	(Jones, Currie et al. 2002)
MySkin (Epithelial cover)	PVC, acrylic acid	Autologous keratinocytes	(Hernon, Dawson et al. 2006)
OrCel (Skin substitute)	Collagen	Allogenic fibroblasts & keratinocytes	(Boyce and Warden 2002)
Pelnac (Skin substitute)	Silicone, collagen	Autologous keratinocytes	(Atiyeh, Hayek et al. 2005)
Permacol (Temporary dermal substitute)	Porcine skin	-	(MacNeil 2007)

Permaderm (Skin substitute)	Bovine collagen	Autologous fibroblasts & keratinocytes	(MacNeil 2007)
PolyActive (Skin substitute)	Polyethylenglycol terepthalate, polybutylene terephthalate	Cultured autologous fibroblasts & keratinocytes	(Boyce and Warden 2002), (Seal, Otero et al. 2001)
Terumo (Skin substitute)	Collagen	Autologous keratinocytes	(Atiyeh, Hayek et al. 2005)
Tissue Tech autograft system (Skin substitute)	Hyaluronic acid ester	Autologous fibroblasts & keratinocytes	(Atiyeh, Hayek et al. 2005)
TissuFleece (Dermal substitute)	Collagen foam	-	(Ng, Khor et al. 2004)
Transcyte (Temporary dermal substitute)	Silicone, nylon mesh, collagen	Neonatal fibroblasts	(Jones, Currie et al. 2002)
VivoDerm (Skin substitute)	Hyaluronan	Autologous keratinocytes	(Ramos-E-Silva and Ribeiro De Castro 2002)

1.4.1 Epidermal replacements.

In 1975 wound management was revolutionised by the development of a technique that used cultured human epidermal cells to form sheets suitable for grafting. This development was soon followed by the use of autologous cultured epidermal-cell grafts for the treatment of burns as well as other acute and chronic wounds (Singer and Clark 1999). The application of cultured epithelial autografts (CEAs) to burn wounds became an extremely useful and often life-saving adjunct in the management of severe burn injuries (Hernon, Harrison et al. In press). Cultured epithelial autografts (CEA's) are produced from confluent multilayer sheets of keratinocytes that have been detached from their culture dishes and attached to a backing dressing to aid the application of the CEA onto the wound bed. CEA's cannot be detached as an integrated sheet generally before nine days but once confluent they must be used within two to three days as beyond this point they begin to blister and detach from

the tissue culture plastic (TCP). Therefore once CEA culture starts plans must be made to use the CEA's within a very narrow window of ten to fourteen days or they lose the ability to attach to the wound bed. This narrow window where the CEA's can be applied means that if the patient's condition changes there can be significant wastage of cells. In a recent study Hernon et al. showed that nearly 50% of CEA's produced were wasted (Hernon, Dawson et al. 2006). However CEA's do provide a wide and permanent skin coverage method (Singer and Clark 1999). More recently several alternative methodologies for delivering keratinocytes to the wound bed have been explored ranging from spraying on cells to delivering cells on collagen coated membranes or chemically defined membranes such as MySkin™ (Hernon, Harrison et al. In press). Wood et al. (2006) reviewed the use of CEA's in the treatment of burn injuries and discussed the main limiting factors in the production of and treatment with CEA's. The time taken to produce the CEA's, the reliability of 'take', vulnerability of grafts, long term durability and the cost implications of treatment were assessed. Whilst the study was inconclusive and unable to answer the question "do CEA's have a role in the treatment of major burns?" it is typical of the variation of opinion found in the literature (Wood, Kolybaba et al. 2006). MySkin™ is one example of a dressing being used in an attempt to overcome some of the limitations encountered in CEA production. Medical grade carrier polymers were treated with an acrylic acid plasma and primary keratinocytes were seeded onto these surfaces and could be cultured in serum free conditions, and transferred from these surfaces onto deepithelialised acellular dermis (Haddow, Steele et al. 2003; Higham, Dawson et al. 2003; Bullock, Higham et al. 2006). The use of this treatment clinically resulted in reduced wastage due to the flexibility in the timing of delivery of cells to the wound bed as the keratinocytes could be transferred to the wound bed whilst subconfluent. This also had the advantage that subconfluent keratinocytes in theory have a greater proliferative potential (Hernon, Dawson et al. 2006).

1.4.2 Dermal replacements and skin substitutes.

The historic standard for rapid closure of full-thickness wounds with a skin substitute is split-thickness, autologous skin applied either as a sheet, or expanded by meshing (Boyce and Warden 2002). Although split-thickness autografts remain the gold-standard they are not always available in sufficient quantity giving rise to the

clinical need for tissue-engineered alternatives (MacNeil 2007). The inclusion of a dermal component in skin substitutes helps prevent wound contraction and provides greater mechanical stability (Singer and Clark 1999). The production of a tissue engineered skin based on human dermis, often supplied by a skin bank, has met with some success with reconstructed skin being used clinically (Chakrabarty, Dawson et al. 1999) and in the laboratory for research purposes (Dawson, Goberdhan et al. 1996; Ghosh, Boyce et al. 1997; Ralston, Layton et al. 1997; Ralston, Layton et al. 1999; Eves, Layton et al. 2000; Chakrabarty, Heaton et al. 2001; Eves, Katerinaki et al. 2003; Haddow, Steele et al. 2003; Higham, Dawson et al. 2003; Harrison, Dalley et al. 2005; Hernon, Dawson et al. 2006; Harrison, Gossiel et al. 2006a; Harrison, Heaton et al. 2006b; Harrison, Gossiel et al. 2006c; Thornton, Harrison et al. Provisionally accepted). Other materials used in the production of skin substitutes include collagen, glycosaminoglycans, hyaluronan, and various polymers (Boyce and Warden 2002; Jones, Currie et al. 2002; Atiyeh, Hayek et al. 2005). Biosynthetic dressings were originally developed to provide temporary coverage to optimise wound healing. They were first introduced in 1979 for the treatment of donor sites (Bello and Falabella 2001).

Allogenic materials such as an allograft from a skin bank can be used as a temporary wound dressing in burns treatment. Human allograft is generally used as a STSG after being procured from organ donors. When used in a viable fresh or cryopreserved state, it vascularises and remains the 'gold standard' of temporary wound closures (Sheridan and Tompkins 2002). An acellular allogenic dermal matrix such as Alloderm, which was approved by the Food and Drug Administration (FDA) in 1992 provides a template with natural dermal porosity for regeneration and allows the use of thinner autografts (Ramos-E-Silva and Ribeiro De Castro 2002). The allogenic dermis still contains its basement membrane proteins and is intended to be combined with a thin epithelial autograft in full thickness burns and chronic wounds (Singer and Clark 1999; Jones, Currie et al. 2002; Ramos-E-Silva and Ribeiro De Castro 2002; Atiyeh, Hayek et al. 2005; Enoch, Grey et al. 2006b). The acellular nature of this product removes the specific immune response and so the risk of rejection but there is still a risk of disease transmission (Ng, Khor et al. 2004). Once applied to the wound bed it becomes repopulated with the patient's own cells, generally revascularises quickly and is incorporated into the healing tissue. It

reportedly has a good take rate and reduces the scarring associated with full thickness wounds (Jones, Currie et al. 2002; Atiyeh, Hayek et al. 2005). Some other commercial dermal matrices used in skin reconstruction are Apligraf, Dermagraft, Integra, and OrCel.

Integra artificial skin is currently the most widely accepted synthetic skin substitute to be developed for use on burns patients (Jones, Currie et al. 2002). The FDA gave approval in 2002 for Integra to be used in "the postexcisional treatment of life-threatening full-thickness or deep partial-thickness thermal injuries where sufficient autograft is not available at the time of excision or not desirable due to the physiological condition of the patient. Integra Dermal Regeneration Template is also indicated for the repair of scar contractures when other therapies have failed or when donor sites for repair are not sufficient or desirable due to the physiological condition of the patient" (Schultz 2002). Integra is a biopolymer tissue-engineered bilayer material consisting of a bovine collagen and shark chondroitin-6-sulfate dermal regeneration template with a temporary silicone epidermal layer (Jones, Currie et al. 2002; MacNeil 2007). The dermal regeneration template is approximately 2 mm thick and has a 70-200 μm pore size that allows for the matrix to become vascularised and for fibroblast invasion (Ramos-E-Silva and Ribeiro De Castro 2002; Sheridan and Tompkins 2002). Neovascularisation usually occurs within 28 days at which point the silicone layer is removed and replaced with a STSG or epidermal autograft (Singer and Clark 1999; Atiyeh, Hayek et al. 2005; MacNeil 2007). The direct application of cells to the dermal matrix was found to be problematic because the cells failed to attach securely (MacNeil 2007). Integra provides immediate permanent coverage for surgically excised full thickness burns and is also used in reconstructive surgery. It requires a healthy and non-infected wound bed (Enoch, Grey et al. 2006b). Case studies show that burn wounds treated with Integra show good cosmetic and functional results with softer scars compared to STSG's (Kremer, Lang et al. 2000; Papp and Harma 2003; Wisser and Steffes 2003; Wisser, Rennekampff et al. 2004).

Apligraf is a bilayer of a neonatal fibroblast populated bovine collagen matrix seeded with neonatal keratinocytes. The exposure of Apligraf to an air-liquid interface

during production allows the formation of a stratified differentiated epithelium (Ehrenreich and Ruszczak 2006). Apligraf is available in a ready to use form with a five day shelf life and is primarily used in the treatment of chronic ulcers (Jones, Currie et al. 2002). It resembles human skin histologically (Ramos-E-Silva and Ribeiro De Castro 2002) and it appears to hasten healing especially in deep and chronic wounds (Jones, Currie et al. 2002). According to Ehrenreich and Ruszczak (2006) Apligraf was initially intended for use in burns treatment. Whilst meshed Apligraf over a meshed autograft did not improve the 'take rate' of the autograft, a better quality scar was achieved and burns treated in this manner were judged to have healed better than those treated with an autograft alone. Despite this promising study Apligraf use in burns is rare as Apligraf is only FDA approved for use in chronic wounds i.e. ulcers and not in burns (Ehrenreich and Ruszczak 2006).

OrCel is composed of type I bovine collagen in which neonatal fibroblasts and keratinocytes are seeded. The fibroblasts are seeded within a porous collagen sponge whilst the keratinocytes are seeded on the nonporous side of the matrix. OrCel is not exposed to an air-liquid interface during production and therefore the epithelium is not terminally differentiated (Ehrenreich and Ruszczak 2006). OrCel is commonly used in acute and chronic deep dermal ulcers, partial thickness burns and donor site wounds. Its main disadvantages are that it cannot be used on infected wounds or on patients allergic to bovine collagen (Enoch, Grey et al. 2006b). According to Supp and Boyce (2005) OrCel is designed for grafting to partial thickness wounds to provide a favourable matrix for host cell migration. OrCel has been indicated for use in the treatment of donor sites in patients with burns, and surgical wounds and donor sites in patients with epidermolysis bullosa (Supp and Boyce 2005). In a recent study comparing two skin equivalents OrCel and Biobrane-L in treating split-thickness donor sites in burns patients OrCel was shown to reduce healing time and scarring (Still, Glat et al. 2003). However, these findings were deemed not clinically meaningful by the FDA (Ehrenreich and Ruszczak 2006).

Wound healing matrices can be produced using synthetic as well as natural materials. One such commercial matrix is Dermagraft which is prepared using cryopreserved neonatal fibroblasts seeded in a bioabsorbable polyglactin mesh and is usually combined with a meshed autograft (Atiyeh, Hayek et al. 2005; Supp and

Boyce 2005). The fibroblasts become confluent among the degrading polymer fibres, producing ECM proteins and secreting growth factors in the interstices of the mesh (Paranteau, Hardin-Young et al. 2000; Seal, Otero et al. 2001; Jones, Currie et al. 2002). Dermagraft has been shown to increase angiogenesis (Naughton 2000) and epithelisation but does not close the wound. It is approved by the FDA for use in full thickness diabetic foot ulcers but not for use in burns and had no significant effect on the 'take' of STSG's when STSG's were placed above Dermagraft in full thickness burns (Ehrenreich and Ruszczak 2006).

Research into new and improved matrices for wound healing is ongoing. Ng and Hutmacher (2006) have produced dermal matrices using synthetic rather than natural materials. Weft-knitted poly(lactic-co-glycolic acid) mesh and starch dialdehyde-crosslinked collagen-hyaluronic acid matrices were seeded with dermal fibroblasts and keratinocytes and were investigated for use as skin equivalents. *In vitro* these showed no sign of contracting. When cultured at an air-liquid interface a stratified keratinocyte layer resembling native epidermis was observed more readily on the starch-collagen-hyaluronic acid matrix than on the poly(lactic-co-glycolic acid) matrix. These matrices were subsequently transplanted onto the backs of rats and the wounds contracted to a similar degree as those treated with autografts with untreated wounds contracting the most (Ng and Hutmacher 2006). El Ghalbzouri et al. (2004) investigated the use of a polybutylene terephthalate / polyethylene oxide terephthalate (PBT/PEGT) (PolyActive) porous film as a dermal scaffold. ECM proteins such as collagen and fibrin could be incorporated later and keratinocytes could be seeded directly onto the matrix or a cultured epithelial sheet added. In serum free conditions at an air-liquid interface a full thickness skin equivalent with a differentiated epidermis could be produced which did not contract during the culture period and was easy to handle (El Ghalbzouri, Lamme et al. 2004).

1.4.3 Dressings.

Dressings have been used since antiquity to aid the healing process. The choice of which dressing to use for a particular wound requires an understanding of wound healing and the properties of the available dressings (Lionelli and Lawrence 2003). Over the past century, our understanding of wound healing has changed. Up until the

mid 1900's it was thought that wounds healed more quickly if they were kept dry and left uncovered. It is now understood that wounds heal faster and better when in a moist environment and occluded wounds have been shown to heal with cosmetically superior results (Menaker 2001). The idea of an occlusive dressing is not new. The Smith Papyrus of 1615 BC states that closed wounds heal more quickly than open wounds and describes the creation of a dressing made from linen strips covered with a gumlike substance. Hippocrates however stated that chronic wounds should not be closed as it was thought that chronic wounds were an indication that something harmful needed to leave the body and this attitude continued until the 19th century (Eaglstein 2001). In the early 1960's two articles were published in Nature (Winter 1962; Hinnman and Maibach 1963) which showed in both a porcine model and in humans that acute, partial thickness excisional wounds experienced significant increases in reepithelialisation rates when the exposed tissues were maintained in a moist local environment by a semi-occlusive polyethylene film as opposed to being allowed to desiccate when uncovered. This lead to the introduction of semi-occlusive dressings for the treatment of chronic wounds in the 1970's (Ovington 2007). Traditionally wet-to-dry gauze has been used to dress wounds. Dressings that create and maintain a moist environment, however, are now considered to provide the optimal conditions for wound healing. Moisture under occlusive dressings not only increases the rate of epithelialisation but also promotes healing through moisture itself and the presence initially of a low oxygen tension (promoting the inflammatory phase). Traditional dressings such as gauze do not exhibit these properties; it may be disruptive to the healing wound as it dries and causes tissue damage when it is removed (Jones, Grey et al. 2006). Occlusive dressings have been shown to be effective in the treatment of donor sites for STSG's (Wiechula 2003) and in the treatment of second degree burns (Field and Kerstein 1994; Wiechula 2003). According to Lionelli and Lawrence (2003) occlusive dressings can be divided into two classes, non-biologic and biologic, with non-biologic dressings further divided into alginates, films, foams, hydrocolloids and hydrogels (Eaglstein 2001; Menaker 2001; Lionelli and Lawrence 2003). The roles of human amnion, alginates, polymer films, polymer foams and hydrocolloids as wound dressings will be discussed here. With the role of hydrogels as wound dressings covered within the section on hydrogels in section 1.5.3.

An example of a biologic dressing is amnion. Since 1910 allogenic amnion has been used as a wound dressing and it has been claimed that it is one of the most effective biological dressings ever used in burn treatment. Its use has special appeal in developing countries particularly where religious barriers preclude the acceptance of bovine, porcine or cadaveric skin (Atiyeh, Hayek et al. 2005). It is used as a temporary dressing for clean superficial wounds such as partial thickness burns, donor sites, and freshly excised burns awaiting donor site availability (Sheridan and Tompkins 2002). Generally fresh amnion is used but according to Maral et al. (1999) the use of frozen, dried, irradiated and lyophilised amnion has also been reported. Whilst amnion has been shown to have low antigenicity, be effective in eliminating pain, allowing wounds to dry faster and in promoting early reepithelialisation there are concerns associated with its use especially with regards to virus and disease transmission (Kane, Tompkins et al. 1996; Maral, Borman et al. 1999; Sheridan and Tompkins 2002; Atiyeh, Hayek et al. 2005).

Alginates have their origin in the naturally occurring calcium and sodium salts of alginic acid found in the family of brown seaweed Phaeophyceae, kelp and algae (Menaker 2001; Ramos-E-Silva and Ribeiro De Castro 2002; Jones, Grey et al. 2006). They are composed of soft non-woven, twisted fibres and non-woven mats (Cho and Lo 1998; Ramos-E-Silva and Ribeiro De Castro 2002; Lionelli and Lawrence 2003). They generally fall into one of two categories, those containing 100% calcium alginate and those containing both calcium and sodium alginate (usually 80:20 Ca:Na) (Jones, Grey et al. 2006). Alginates also contain galuronic and mannuronic acid. The amounts of these two compounds in the gel alter the material properties with high levels of galuronic acid resulting in firmer gels which maintain their shape. Dressings containing high amounts of mannuronic acid are correspondingly weaker (Ramos-E-Silva and Ribeiro De Castro 2002). Alginate gels release calcium ions and have haemostatic properties (Menaker 2001). According to Cho and Lo (1998) when the alginate dressing is applied, sodium from the wound exudate and calcium from the alginate fibres undergo an ion exchange reaction, forming a soluble sodium alginate gel. The free calcium ions produced during the ion exchange reaction act to amplify the clotting cascade, according the alginate dressings significant haemostatic properties (Cho and Lo 1998). The soluble sodium alginate gel produced during the ion exchange reaction creates an occlusive

environment that aids wound healing (Lionelli and Lawrence 2003). Alginates are best used in wounds with a moderate to heavy wound exudate and in granulating and reepithelialising wounds where some exudate is present. They are especially useful in packing exuding wounds and rope alginates are easy to apply. Several alginates have good wet strength and can be removed in one piece thus reducing trauma to the wound. However a second, protective dressing is always required and there is a risk of the dressing drying the wound bed and therefore alginate dressings are not recommended for wounds with a low volume of exudate (Eaglstein 2001). Alginate dressings have been successfully used in chronic ulcers, partial and full thickness burns and STSG's (Cho and Lo 1998). Several alginate dressings are available commercially such as Algiderm, Algisite, Algosteril, Carasorb, Curasorb Calcium Alginate Dressing, Kalginate, Kaltostat, Melgisorb, SeaSorb, Sorbsan, Tegagen, Urgosorb, (Menaker 2001; Ramos-E-Silva and Ribeiro De Castro 2002; Lionelli and Lawrence 2003; Jones, Grey et al. 2006; Ovington 2007).

Polymer films used as wound dressings especially in the early stages of burns treatment can be as simple as Clingfilm (polyvinylchloride sheeting) which would normally be found covering food in a kitchen (Benson, Dickson et al. 2006). Film dressings are typically clear polyurethane or copolyester membranes, usually about 0.2mm thick coated on one side with an adhesive (Cho and Lo 1998; Menaker 2001; Ramos-E-Silva and Ribeiro De Castro 2002; Lionelli and Lawrence 2003). According to Cho and Lo these dressings are highly elastic and transparent, allowing continuous inspection of the wound. They are semi occlusive dressings as they are permeable to oxygen, carbon dioxide and water vapour but are impermeable to wound fluids and bacteria (Cho and Lo 1998). Film dressings have little absorptive capacity, so frequent dressing changes are needed (Menaker 2001). Unfortunately they can adhere to wounds and so retard wound healing by removing new epithelium during dressing changes (Cho and Lo 1998; Menaker 2001). Film dressings are best used in donor sites, shallow wounds, superficial burns, partial thickness wounds with minimal exudate and abrasions (Eaglstein 2001; Menaker 2001) Commercially available film dressings include Acu-Derm, Bioclusive Transparent Dressing, Blisterfilm Transparent Dressing, Carrafilm, Hi/Moist Transparent, Mefilm, Omniderm, Opsite Wound Dressing, Polyskin II Transparent Dressing, Silon TSR (temporary skin replacement), Tegaderm Transparent Dressing, Transeal, Transite

Exudate Transfer Film, Transparent Adhesive, Uniflex Transparent Dressing, Visi Derm II by Medline (Cho and Lo 1998; Eaglstein 2001; Ramos-E-Silva and Ribeiro De Castro 2002; Lionelli and Lawrence 2003).

Foam dressings tend to be composite materials. They are usually composed of a polyurethane or silicone foam mesh inner layer and an outer semi permeable membrane of polyurethane, polyester, silicone, or Gore-Tex surrounding a polyoxyethylene glycol foam (Cho and Lo 1998; Ramos-E-Silva and Ribeiro De Castro 2002; Jones, Grey et al. 2006). Early foam dressings were custom polymerised or cut for individual wounds. Foam dressings are both absorbent and nonadherent, they are permeable to both water and gases and they can expand and conform to wounds with unusual proportions. They are comfortable and can be removed easily for cleaning (Cho and Lo 1998; Lionelli and Lawrence 2003). They aid the dispersion of wound exudate throughout the absorbent layer and the presence of the semi permeable backing dressing prevents leakage (Jones, Grey et al. 2006). Disadvantages of polymer foams as wound dressings includes their opacity so the wound cannot be visualised, additional dressings are required to secure the foam, the dressing needs to be changed every three days, they cannot be used on dry wounds and they can become incorporated into the wound (Cho and Lo 1998; Menaker 2001; Ramos-E-Silva and Ribeiro De Castro 2002). Foam wound dressings are usually used in heavily exudating and partial thickness wounds, especially during the early inflammatory phase following debridement and sloughing when drainage is at its peak, they are also used as pressure relief, packing in deep cavity wounds and in venous leg ulcers (Eaglstein 2001; Menaker 2001). Some commercially available foam dressings include Allevyn, Cutinova plus, Flexzan, Flexzan Extra, Optifoam, PolyMemn, Revitaderm, Silastic foam, Sof-Foam and Tielle (Eaglstein 2001; Menaker 2001; Ramos-E-Silva and Ribeiro De Castro 2002; Ovington 2007).

The term hydrocolloid is used to describe a family of dressings containing a hydrocolloid matrix composed of such materials as gelatin, pectin, and carboxymethylcellulose (Lionelli and Lawrence 2003). Hydrocolloid dressings are compound formulations usually composed of an outer polyurethane foam bonded to a middle hydrocolloid gelling agent, usually sodium carboxymethylcellulose, and an inner adhesive layer (Cho and Lo 1998; Ramos-E-Silva and Ribeiro De Castro

2002). Hydrocolloid dressings are available as adhesive wafers, sheets or as pastes or powders and were derived from materials used initially as ostomy barrier products (Cho and Lo 1998; Lionelli and Lawrence 2003; Jones, Grey et al. 2006). Hydrocolloid dressings are impermeable to oxygen, carbon dioxide, water vapour and bacteria (Ramos-E-Silva and Ribeiro De Castro 2002; Lionelli and Lawrence 2003). Other advantages include enhanced angiogenesis and an ability to conform to wound contours (Menaker 2001). The dressing absorbs wound exudate well, and upon absorbing the exudate the matrix swells and liquefies forming a viscous, colloidal gel that prevents adherence of the dressing to the wound bed (Cho and Lo 1998; Lionelli and Lawrence 2003). Hydrocolloid dressings are used in wounds draining low to moderate amounts of exudate, burns, partial or full thickness wounds and pressure ulcers. They have also been used in skin disorders such as psoriasis and epidermolysis bullosa and are used to prevent blisters in athletes and diabetics (Cho and Lo 1998; Eaglstein 2001; Menaker 2001). Hydrocolloid dressings are particularly good for use on hands and other small areas of superficial or partial thickness burns, although they leave a 'skim' of exudate that needs to be removed to allow appropriate assessment of the wound (Benson, Dickson et al. 2006). Hydrocolloids cannot be used in wounds with a moderate to heavy exudate, they are impermeable to oxygen, and they can break down resulting in time consuming removal of the hydrocolloid residue from the wound (Eaglstein 2001). They should not be used on infected wounds and are opaque making visualisation of the wound difficult (Ramos-E-Silva and Ribeiro De Castro 2002). Some commercially available hydrocolloid dressings include Alione, CombiDERM, CombiDERM N, Comfeel, Comfeel Plus, Cutinova Hydro, Cutinova Thin, DuoDERM, Extra Thin, Granuflex, Hydrocol, Hyprapad, Intact, Intrasite wound dressing, J & J ulcer dressing, NuDerm, Orahesive, RepliCare, Restore, Sween-A-Peel, Tegasorb, Tegasorb Thin and Ultec (Cho and Lo 1998; Eaglstein 2001; Lionelli and Lawrence 2003; Jones, Grey et al. 2006; Ovington 2007).

Wound dressings continue to develop with more coming onto the market annually, a recent study states that in a recent issue of a wound care products buyer's guide lists more than 400 individual advanced wound dressings including 25 alginates, 55 foams, 50 hydrocolloids, 51 hydrogels and 24 transparent films (Ovington 2007). Many specialist dressings are available especially for burns wounds. However, the

prevention of infection is a major problem encountered in wound healing. Systemically administered antibiotics should be reserved for treating invasive infection whilst topical antibacterials are used for superficial, local management of an open wound surface. Antibacterials have been used for centuries and are still in widespread use (Leaper 2006). Antimicrobials are among many additives being incorporated into wound dressings to aid healing. According to Leaper, the introduction of silver into wound care as an antibacterial, particularly in burns is relatively recent. There is a growing number of silver dressings commercially available these include creams such as Flamazine, foams, hydrogels, hydrocolloids and polymeric films and meshes (Hudspith and Rayatt 2004). Dressings containing the nanocrystalline form of silver appear to have the best evidence of consistency in relation to clinical outcomes. These dressings significantly reduce the bacterial burden in chronic wounds and there may be further benefit in reducing infection in the management of burns (Leaper 2006). Silver has been incorporated into poly(ethylene glycol) hydrogels. This study showed that the multifunctional properties of silver allowed for a simplified polymer that was biocompatible, as showed by *in vitro* testing with fibroblasts and keratinocytes and was still antimicrobial (Babu, Zhang et al. 2006). Another antimicrobial incorporated into wound dressings is poly(vinylpyrrolidinone)-iodine (PVP-I). Vogt et al. (2006) produced a PVP-I hydrosome and hydrogel which was shown to prevent infection, promote wound healing, improve the quality of healing and increase graft 'take' in smokers and on wounds with increased bacterial load. The incorporation of curcumin, an antioxidant, into collagen gels for use as wound dressings has also been recently shown to increase cell migration towards the wound and so speed up the wound healing cascade of inflammation, proliferation and scar formation (Gopinath, Ahmed et al. 2004). EGF has also been successfully incorporated into chitosan gels to improve wound healing in burns (Alemdaroglu, Degim et al. 2006).

1.5 Hydrogels.

Hydrogels as defined by Peppas and Mikos (1986) are water-swollen networks (crosslinked structures) of hydrophilic homopolymers and copolymers. They are three-dimensional and the crosslinks can be formed by covalent or ionic bonds. Often, weaker forces such as van der Waals forces and hydrogen bonds can serve as

crosslinks, thus forming swollen networks which behave as hydrogels. Finally, semi-crystalline, uncrosslinked hydrophilic polymers may form hydrogels upon swelling since the crystallites act as physical crosslinks and do not dissolve in water (Peppas and Mikos 1986). Hydrogels may absorb from 10-20% (an arbitrary lower limit) up to thousands of times their dry weight in water (Hoffman 2001). Hydrophilic polymers include poly(ethylene oxide), poly(acrylamide), poly(ethylene glycol) or poly(vinylpyrrolidinone) (Ramos-E-Silva and Ribeiro De Castro 2002). According to Peppas et al. (2006) the polymer engineer can design and synthesise polymer networks with molecular-scale control over structure such as crosslinking density and with tailored properties, such as biodegradation, mechanical strength, and chemical and biological response to stimuli (Peppas, Hilt et al. 2006). Physical hydrogels can be formed in many ways, these include warming or cooling of polymer solutions to form gels and changing the pH of polymer solutions to form gels (Hoffman 2001; Hoffman 2002). Chemical hydrogels can also be formed in a variety of different methods. Polymer solids or solutions can be crosslinked with radiation, chemical crosslinkers or multi-functional reactive compounds. Monomers and crosslinkers can be copolymerised as can monomers and multifunctional macromers. Interpenetrating networks can be formed via the polymerisation of a monomer within a different solid polymer and hydrophobic polymers can be converted chemically to hydrogels (Hoffman 2001; Hoffman 2002).

1.5.1 Polymer synthesis.

By far the most common method of network synthesis involves conventional free radical polymerisation (Patrickios and Georgiou 2003). Free radical polymerisation reactions can be separated into three stages initiation, propagation and termination reactions. According to Cowie (1991) a free radical is an atomic or molecular species whose normal bonding system has been modified such that an unpaired electron remains associated with the new structure. The radical is capable of reacting with an olefinic monomer to generate a chain carrier which can retain its activity long enough to propagate a macromolecular chain under the appropriate condition (Cowie 1991). The distinguishing characteristic of chain polymerisation, where the reactive species is a free radical, cation or anion, is that polymer growth takes place by monomer reacting only with the reactive centre. By far the most common

example of chain polymerisation is that of vinyl monomers and this process can be depicted in the following manner modified from Odian (2004).
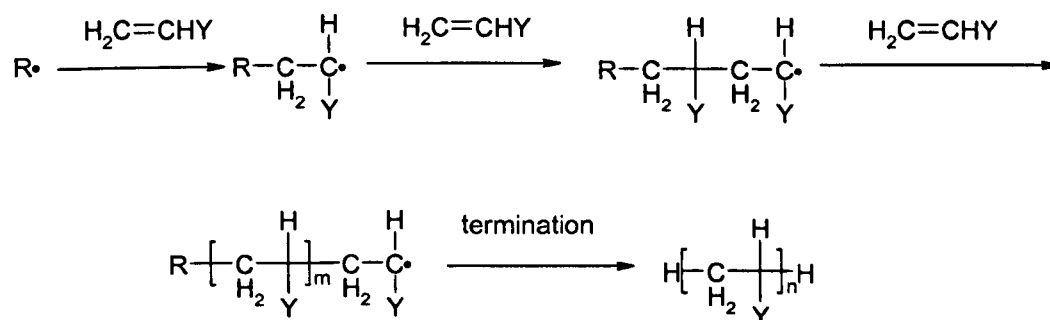
Each monomer molecule that adds to a reactive centre regenerates the reactive centre. Polymer growth proceeds by the successive additions of hundreds or thousands or more monomer molecules. The growth of the polymer chain ceases when the reactive centre is destroyed by one or more of a number of possible termination reactions (Odian 2004). Typically free radical polymerisation reactions can be carried out in one of four different ways 1) with monomer only (*in bulk*), 2) in a solvent (*in solution*), 3) with monomer dispersed in an aqueous phase (*in suspension*) and 4) or as an *emulsion* (Cowie 1991).

An initiator is usually used to initiate free radical polymerisation reactions. Initiators are susceptible to homolytic fission when exposed to heat, electromagnetic radiation or a chemical reaction. α,α'-azobisisobutyronitrile (AIBN) is a commonly used initiator that decomposes when exposed to either electromagnetic radiation (360 nm) or heat ($\approx 60°C$) as follows, reaction scheme taken from Cowie (1991)
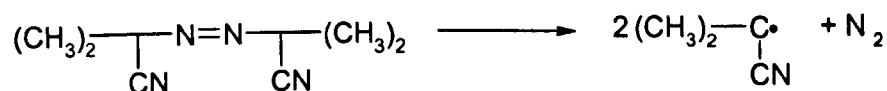
The reaction then propagates in a stepwise manner with molecules adding onto the growing chain. Initially these will be monomers but as the reaction progresses dimers, trimers and larger macromers will be added. In theory the chain should continue to grow in this manner until all the monomer in the system has been used up. However free radicals are very reactive and interact quickly to form inactive covalent bonds. This produces short chains in systems containing a high

concentration of initiator and long chains in systems containing a low concentration of initiator. Chain growth can terminate in one of three ways. Termination 1) via the interaction of two active chains, 2) the reaction of an active chain end with an initiator radical or 3) termination by transfer of the active centre to another molecule which may be solvent, initiator, monomer, inhibitor or an impurity i.e. oxygen. Termination via the interaction of two active chains can occur in one of two ways, by the combination or by disproportionation. Termination by combination is where two chain ends combine to form one long chain. Termination by disproportionation is via hydrogen abstraction from one end to give an unsaturated group and two dead polymer chains Cowie (1991). These principles hold true for both homopolymers and copolymers.

Copolymers are produced when more than one monomer is used during the synthesis. There are five types of copolymers, statistical, random, alternating, block or graft copolymers, some of these are illustrated below where M_1 and M_2 are different monomers (Odian 2004).

Statistical copolymers – there is a statistical distribution of one monomer within the other.

$M_1 + M_2 \rightarrow \sim M_1M_2M_2M_1M_2M_2M_2M_1M_1M_2M_2M_1M_1M_2M_1M_1M_1M_2M_2M_1M_1\sim$

Alternating copolymers – the two monomers alternate along the polymer chain.

$M_1 + M_2 \rightarrow \sim M_1M_2M_1M_2M_1M_2M_1M_2M_1M_2M_1M_2M_1M_2M_1M_2M_1M_2M_1\sim$

Block copolymers – there are blocks of both monomers in the polymer chain.

$M_1 + M_2 \rightarrow \sim M_1M_1M_1M_1M_1M_2M_2M_2M_2M_2M_1M_1M_1M_1M_1M_2M_2M_2M_2M_2M_1\sim$
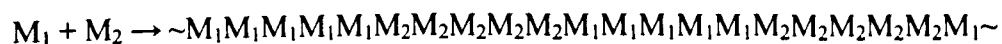
Hydrogels are called permanent or chemical gels when they are covalently crosslinked networks (Hoffman 2001). Diene monomers are often used in copolymerisation reactions to obtain a crosslinked structure. When crosslinking occurs in the copolymerisation it depends on the relative reactivity of the two double bonds of the diene. The extent of crosslinking depends on the latter and on the

amount of diene relative to the other monomer. Several different cases can be distinguished depending on the type of diene. In most instances it is assumed that the diene is present at low concentrations (Odian 2004). When a crosslinked polymer is produced the dimensional stability is improved, the creep rate is lowered, it becomes less prone to heat distortion as the glass transition temperature (Tg) is raised and the resistance to solvents is increased (Cowie 1991). If the selected crosslinker is susceptible to degradation then it is possible to design polymers with predictable degradation profiles. These polymers are especially useful in drug delivery and tissue engineering applications. Controlling degradation behaviour has been one of the critical issues in general biomaterials research. Generally biomaterials need to be cleared from the body once they complete their roles in the body, and degradable materials could be ideal for this purpose. Two approaches are typically used to obtain degradable polymers and hydrogels. In the first, gelling polymers are designed such that their backbone is degradable by hydrolysis and / or enzymatic action. The second approach involves the introduction of degradable crosslinking points to systems that are comprised of non-degradable polymer chains (Lee, Bouhadir et al. 2004). Whilst degradable polymers are usually viewed as superior materials, they have to be approached with caution. The effects of the degradation products i.e. low molecular weight oligomers, charged molecular species or larger pieces of polymer, on surrounding tissues have to be evaluated. Changes in pH associated with the degradation of polymers such as poly(lactic acid) or poly(glycolic acid) can also have an adverse effect on the viability of surrounding tissues. The rates of degradation also have to be evaluated and the effects of the degradation on the material properties of the polymer. A rapidly degrading polymer, which lost the majority of its strength within a day or two, would not be suitable for a wound dressing which may need to stay in place for a week.

When a greater degree of control is required over the final product i.e. when the molecular weight of the polymer needs to be well defined, living polymerisation routes can be used. These include atom transfer radical polymerisation (ATRP) and reversible addition fragmentation transfer polymerisation (RAFT) (Odian 2004). Living radical polymerisation allows the preparation of polymers with predetermined molecular weights, a narrow molecular weight distribution and tailored architecture (e.g. end-functional, block, star) and therefore offers a vast range of new and

advanced materials (Rizzardo, Chiefari et al. 2000). The molecular weights are predetermined by the reagent concentrations and conversion. The polymer products can also be reactivated for chain extension or block copolymer synthesis (Moad, Mayadunne et al. 2003).

1.5.2 Hydrogels as biomaterials.

Hydrogels are appealing for biological applications because of their hydrophilic character and potential for biocompatibility. In the last couple of decades, hydrogels have attracted a great deal of attention, and significant progress has been made in designing, synthesising, and using these materials for many biological and biomedical applications (Peppas, Hilt et al. 2006). Many biomaterials have been designed to meet particular biological and chemical requirements (e.g. biocompatibility, degradability, mediation of cell adhesion, etc.). Other design parameters, such as the physical properties of the biomaterial, were regarded with respect to the processing conditions, the mechanical load capacity, or the diffusivity of solutes, but not with respect to the biological response (Brandl, Sommer et al. 2007).

Neutral synthetic polymers can be generated from derivatives of poly(hydroxyethyl methacrylate) (PHEMA), poly(ethylene glycol) (PEG), poly(vinyl alcohol) (PVA) and poly(N-vinylpyrrolidinone) (PNVP). Polymers that respond to stimuli such as changes in temperature or pH can also be produced. Responsive polymers include poly(acrylic acid) (PAA) which is sensitive to changes in pH and polymers based on poly(N-isopropylacrylamide) (PNIPAAm) which is sensitive to changes in temperature. (Peppas, Hilt et al. 2006).
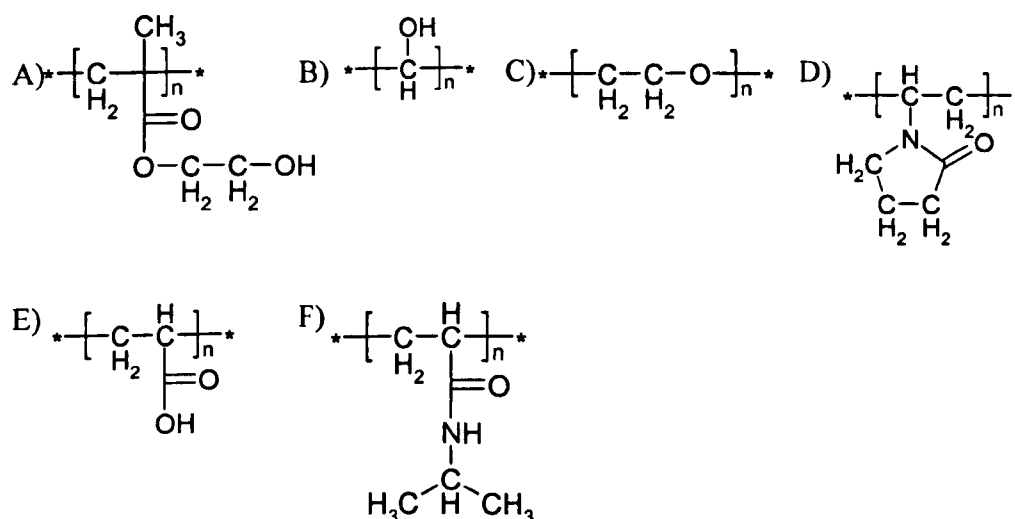
Figure 1.4 Representative chemical structures for some synthetic polymers. Neutral polymers A) poly(hydroxyethyl methacrylate) (PHEMA), B) poly(vinyl alcohol) (PVA) C) poly(ethylene glycol) (PEG) and D) poly(*N*-vinylpyrrolidinone) (PNVP). Responsive polymers E) poly(acrylic acid) (PAA) and F) poly(*N*-isopropylacrylamide) (PNIPAAm).

Hydrogels made from PEG and HEMA are normally neutral and relatively inert to cell attachment (Schneider, English et al. 2004). PEG hydrogels are one of the most widely studied and used biomaterials. According to Peppas et al. (2006) PEG hydrogels are non-toxic, non-immunogenic, and approved by the US FDA for various clinical uses. In many cases PEG has been applied as a 'stealth material' to render materials invisible to biological molecules i.e. proteins (Peppas, Hilt et al. 2006).

PHEMA hydrogels are also widely used in biomedical applications. PHEMA is a common constituent in contact lenses. According to Hoffman (2001) the hydrogel pioneer was a PHEMA hydrogel crosslinked with ethyleneglycol dimethacrylate (EGDMA). These hydrogels were first reported for biomedical applications in the journal Nature in 1960 and have since been successfully used as soft contact lenses. The original PHEMA contact lens was developed by Otto Wichterle in 1961 and contained 38% water, had excellent wetability and was much more comfortable to wear than the hard contact lenses. These contact lenses were subsequently modified by the addition of various hydrophilic monomers such as PNVP and glycerol methacrylate (GMA) (Lloyd, Faragher et al. 2001). HEMA has been copolymerised with other monomers such as methyl methacrylate to modify the gels material

properties i.e. the swelling and mechanical properties. These approaches have lead to PHEMA being used in drug delivery and tissue engineering applications (Peppas, Hilt et al. 2006). PHEMA based hydrogels have also been used in wound healing applications as wound dressings and skin substitutes and they have also been used in cartilage tissue engineering (Seal, Otero et al. 2001).

Schneider et al. (2004) modified the charge of PHEMA and PEG hydrogels to produce positively and negatively charged gels as well as the normal neutral gels. 2-sulfoethyl methacrylate was incorporated into the hydrogels to produce negatively charged gels. 2-methacryloxy ethyltrimethyl ammonium chloride was added to produce positively charged hydrogels. Gels were produced with fixed charges of 200, 0 and +200 mM. GRGDS (Glycine - Arginine - Glycine - Aspartic acid – Serine) adhesion sites were also covalently bound to the hydrogel surfaces. This study showed that murine MC3T3-E1 osteoblasts and 3T3 fibroblasts preferred to attach to the positively charged surfaces instead of the negatively charged and neutral surfaces. The osteoblastic cells attached better to both gels than the fibroblastic cells. Both cell types preferred the HEMA gels than the PEG gels and the cells attached better to the positively charged gels than to the gels with GRGDS grafted to the surface (Schneider, English et al. 2004). This contrasts with the work of Chen et al. (2005) who showed that bovine foetal aorta endothelial cells preferentially attached and proliferated on negatively charged PAA, poly(sodium p-styrene sulfonate) (PNaSS) and poly(2-acrylamido-2-methyl-1-propanesulfonic sodium) (PNaAMPS) hydrogels with zeta potentials between -20 mV and -35 mV over the neutral PVA and poly(acrylamide) (PAAm) hydrogels (Chen, Shiraishi et al. 2005). Soltys-Robitaille et al. (2001) also demonstrated a link between the surface charge of contact lenses and protein adsorption. Cationic proteins such as lysozyme adsorbed preferentially to anionic polymers and anionic proteins such as human serum albumen adsorbed preferentially to cationic polymers. Nonionic polymers showed no detectable protein adsorption (Soltys-Robitaille, Ammon et al. 2001). Kulik and Ikada (1996) showed that hydrogel charge also affected the adsorption of platelets to various hydrogels. They showed that platelets adsorbed preferentially onto the surfaces of cationic polymers, and reduced adsorption was seen on anionic polymers. This was explained by the anionic nature of the platelet cell membrane. Platelet adsorption was also reduced on polymers with large water contents i.e.

greater than 90% (Kulik and Ikada 1996). Nuttelman et al. (2001) showed that the water content of hydrogels affected the ability of proteins, specifically fibronectin to adhere to the hydrogel surface. This lack of protein adhesion made cell attachment difficult and therefore fibronectin was chemically attached to the surface of the PVA hydrogel used in this study to allow NIH3T3 fibroblasts to attach and migrate (Nuttelman, Mortisen et al. 2001). The link between the water content of hydrogels and cell attachment has also been illustrated by Haigh et al. (2002) and Rimmer et al. (2005).

It appears that a number of factors affect the adhesion of cells to polymers namely the charge associated with the polymer surface, the water content of polymers and the cells themselves. It has been shown that cells cannot attach directly to polymers. Proteins such as fibronectin, laminin or vitronectin must be adsorbed to the polymers surface before cells can attach (Nuttelman, Mortisen et al. 2001). If the water content of the polymer is too high then the protein will not be able to attach to the surface as the surface will not be 'seen' by the protein. The adage of opposites attract is also true when looking at the adsorption of proteins to polymers with charged proteins adsorbing onto the surfaces of polymers with the opposite charge. Proteins not only need to be adsorbed onto the polymer but they need to be the correct proteins for cell attachment. According to Nuttelman et al. (2001) the driving force for protein adsorption to hydrophilic surfaces is enthalpic, conversely the driving force for protein adsorption to hydrophobic surfaces is entropic, and protein adsorption to hydrophobic surfaces is usually irreversible, opposed to the reversible adsorption of proteins to hydrophilic surfaces. Nuttelman et al. (2001) also states that maximal cell adhesion to polymeric surfaces occurs on surfaces of moderate water wetability. Surfaces of high or low water wetabilities discourage cell adhesion. At this time it is impossible to decide which is the more important of the material properties i.e. surface charge or water content when it comes to cell attachment to polymers.

1.5.2.1 Poly(vinylpyrrolidinone)

Poly(vinylpyrrolidinone) (PNVP) which is sometimes known as poly(vinylpyrrolidone) or povidone is the water-soluble homopolymer of N-vinyl-2-pyrrolidone. PNVP was first developed in Germany at I.G. Farben by Professor

Walter Reppe and his colleagues during the 1930's, PNVP was subsequently widely used as a blood-plasma substitute and extender during world war II (Robinson, Sullivan et al. 1990). However in 1978 the FDA determined that the use of PNVP in normal saline was unsafe for use as a plasma expander due to the risk of accumulation in the body causing the formation of granulomas. The FDA also stated that Povidone also interferes with blood coagulation, haemostasis, and blood typing and cross matching (FDA 1998). PNVP still has a wide range of use in biomedical applications especially in the pharmaceutical industry where it is used as a binder for tablets with the World Health Organisation (WHO) granting an acceptable daily intake of 0-50 mg.kg^{-1}.day^{-1} for PNVP (Robinson, Sullivan et al. 1990).

PNVP hydrogels are widely used in biomedical applications. PNVP is a common constituent in contact lenses where it is used to raise the water content of the lens (Lloyd, Faragher et al. 2001). Copolymers of PNVP have been investigated as adhesives for surgical wounds (Kao, Manivannan et al. 1997), wound dressings (Risbud, Hardikar et al. 2000; Razzak, Darwis et al. 2001; Vogt, Hauser et al. 2001; Wu, Bao et al. 2001; Lopergolo, Lugao et al. 2002; Lugao, Rogero et al. 2002; Ajji, Othman et al. 2005; Sen and Avci 2005; Vogt, Reimer et al. 2006) and for drug delivery applications (Berscht, Nies et al. 1994; Liu, Fullwood et al. 2000; Liu and Rimmer 2002a; Liu and Rimmer 2002b; D'Souza, Schowen et al. 2004; Kaneda, Tsutsumi et al. 2004; Kodaira, Tsutsumi et al. 2004; Bajpai and Dubey 2005; Devine, Devery et al. 2006; Saxena, Mozumdar et al. 2006). PNVP is commonly encountered as an antiseptic when complexed with Iodine under the trade name Betadine. PNVP hydrogels have also been investigated by the group of T.V. Chirila for use as a vitreous substitute (Vijayasekaran, Chirila et al. 1996; Chirila, Hong et al. 1998; Hong, Chirila et al. 1998). These studies showed that PNVP hydrogels had the required material properties i.e. these hydrogels were injectable, had the correct optical properties and did not stimulate an excessive immune response (Vijayasekaran, Chirila et al. 1996; Hong, Chirila et al. 1998). However *in vitro* cytotoxicity testing showed that these PNVP based hydrogels could significantly increase fibroblast viability both in the presence and absence of serum. The extent to which cell viability altered when exposed to PNVP was affected by the crosslinkers used in the study (Hong, Chirila et al. 1997). The ability of PNVP based hydrogels to increase cell viability was confirmed by Risbud et al. (2000) who showed that cell

viability could be significantly increased when cells were grown on PNVP-chitosan hydrogels.

PNVP is a common constituent in wound dressings, its extreme hydrophilicity means that cells find adhesion difficult. It has also been postulated that PNVP could be used in the place of poly(ethyleneglycol) (PEG) due to their similar "stealth" properties especially when coating nanoparticles for infiltration into the body (Saxena, Mozumdar et al. 2006). A series of PNVP polymers have been investigated by the group of T.V. Chirila for use as vitreous substitutes. During cytotoxicity testing of these polymers a growth promoting effect was observed. This was especially potent when 3T3 Swiss mouse fibroblasts were cultured with these polymers in the absence of serum, this was also shown to be dependent on the concentration of polymer in the medium (Hong, Chirila et al. 1997). Gels crosslinked with Diethyleneglycol Dimethacrylate (DEGDMA) performed best in cytotoxicity testing. Cytotoxicity was assessed using the SRB assay, an assay for cell protein content. For all of the polymers the equilibrium water contents were between 95-98%. The polymers had material properties suitable for use as vitreous substitutes and for injecting through a large gauge needle (Hong, Chirila et al. 1997). *In vivo* testing showed that PNVP was still present in the eyes 4 weeks after operation, the eyes however showed signs of giant cell infiltration (Vijayasekaran, Chirila et al. 1996). Risbud et al. (2000) showed that poly(vinylpyrrolidinone-chitosan) hydrogels could increase the viability and proliferation, assessed by MTT-ESTA assay and image analysis, of both fibroblasts and SiHa epithelial cells. Medium that had been incubated in the presence of these polymers was also shown to increase cell viability. In the case of the SiHa cells the more conditioned medium was present, up to 30%, the greater the increase in viability. For fibroblasts at concentrations of 10 and 20% conditioned medium and increase in viability above the tissue culture plastic (TCP) control was seen, however once the concentration reached 30% the viability of the cells, whilst still greater than the control cells, dropped significantly from that of the cells cultured in 20% conditioned medium.

PNVP has also been investigated for use as a vitreous substitute (Vijayasekaran, Chirila et al. 1996; Hong, Chirila et al. 1997; Chirila, Hong et al. 1998; Hong,

Chirila et al. 1998). *In vitro* biodegradation experiments indicated that there should be no biodegradation over the 4 week examination period. Further *in vivo* testing showed that 50% of the injected crosslinked hydrogel disappeared from the eye while over the same period 80% of the homopolymer was lost within the same period (Hong, Chirila et al. 1998). This loss of polymer could be a major concern. However PNVP has been shown to induce only minor storage-related functional changes to organs and this is influenced by molecular weight. Linear PNVP's with molecular weights of less than 70 kDa passively excreted by the kidneys (Chirila, Hong et al. 1998). Other biodegradation studies (Bruining, Koole et al. 1999) have shown that PNVP crosslinked with a novel carbonate crosslinker (figure 1.5) , with a crosslinker:NVP mole ratio of 1:10.5 was still stable after a week. However with decreasing crosslinker concentrations the degradation rate increased 1:105 degraded in less than 1 day and 1:1000 degraded immediately at 37°C in phosphate buffered saline. There are no literature studies showing the degradation of P(NVP-co-DEGBAC) hydrogels. However the similarities between the two crosslinkers suggest that the degradation profiles may be similar. The structure of DEGBAC is shown in figure 1.6
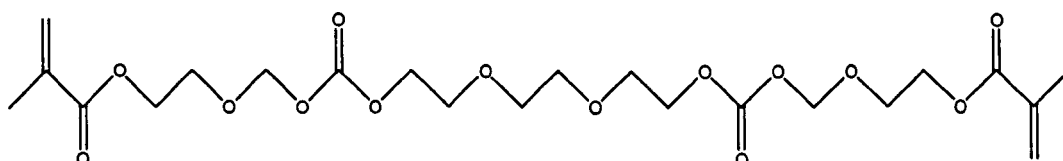
Figure 1.5 Novel carbonate crosslinker (Bruining, Koole et al. 1999)
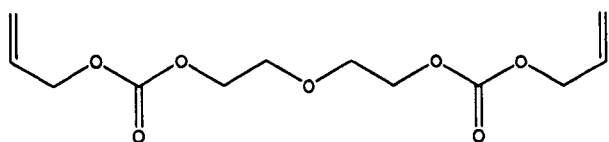
Figure 1.6 Structure of Diethylene glycol bisallylcarbonate

The material and chemical characterisation of poly(vinylpyrrolidinone), of both the homopolymer and various copolymers are well documented in the literature. The ^{13}C-NMR spectrum of uncrosslinked poly(vinylpyrrolidinone) in methanol-d_4 is shown in 1.7.
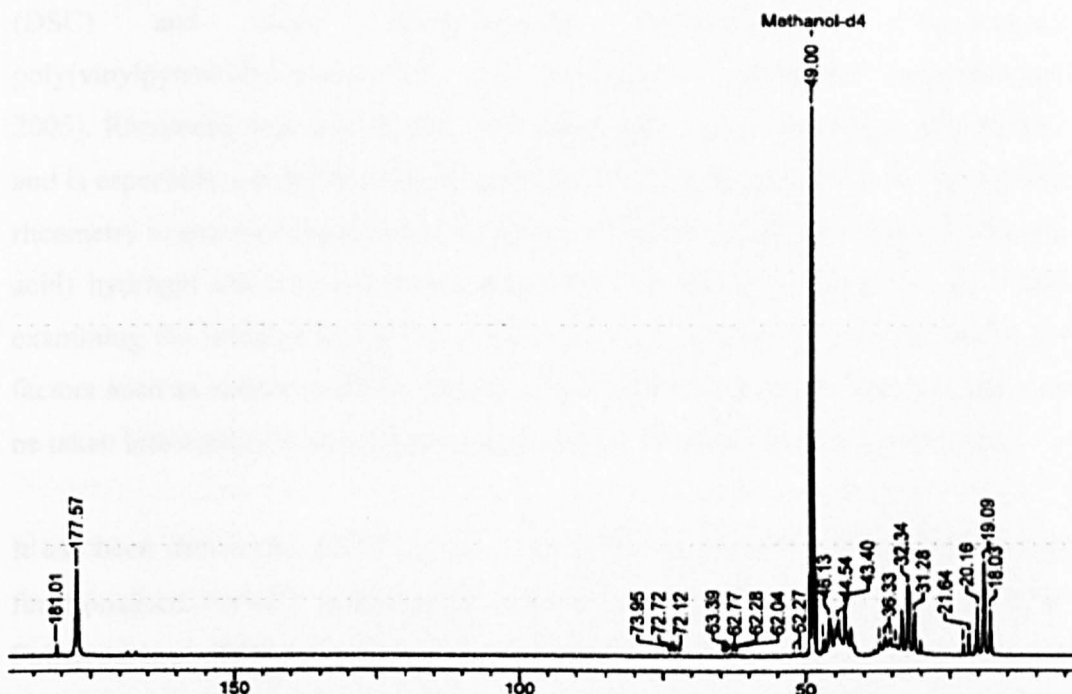
Figure 1.7 ^{13}C-NMR spectrum of Plasdone C-15 in methanol – d_4. A commercially available linear homopolymer of poly(vinylpyrrolidinone) with molecular weight 10^4 Da (Raith, Kuhn et al. 2002).

Material characterisation of various poly(vinylpyrrolidinone) copolymers for various applications has been approached in different ways. Dynamic mechanical analysis (DMA) and differential scanning calorimetry have been used to probe the properties of poly(vinylpyrrolidinone)-poly(vinyl alcohol) blends (Cauich-Rodriguez, Deb et al. 1996a; Cauich-Rodriguez, Deb et al. 1996b) giving storage moduli, glass transition temperatures (T_g) and stress-strain (σ-ε) behaviour. Equilibrium water content (EWC) measurements are commonly used to characterise hydrogels as the hydrophilicity or hydrophobicity of these polymers is important when polymers are considered for biomedical applications. Shantha et al. (2003) investigated how increasing N-vinyl pyrrolidinone content in a series of poly(lactose acrylate-N-vinyl-2-pyrrolidinone) hydrogels and increasing crosslinker concentration affected the EWC of these hydrogels. Uniaxial tensile and compression testing of hydrogels is also possible. Poly(vinylpyrrolidinone)-gelatin hydrogels have been tested using

compression testing (Lopes and Felisberti 2003) whilst other hydrogels have been tested using specimens cast into dumbbell moulds for uniaxial tensile testing (Hinkley, Morgret et al. 2004). Swelling studies and Fourier transform infrared (FTIR) spectroscopy have been used along with differential scanning calorimetry (DSC) and other chromatography techniques to characterise poly(vinylpyrrolidinone-co-acrylic acid) hydrogels (Devine and Higginbotham 2005). Rheometry can also be used to examine the material properties of polymers and is especially suitable for soft polymers like hydrogels. Devine et al. (2005) used rheometry to examine the material properties of a poly(vinylpyrrolidinone-co-acrylic acid) hydrogel allowing the shear modulus (G) to be calculated. However when examining the material properties of hydrogels, as with any polymer, a number of factors such as rubber elasticity theory, viscoelasticity and creep behaviour have to be taken into account when designing experiments (Anseth, Bowman et al. 1996).

It has been shown that PNVP can be conjugated to a number of drugs to produce functionalised PNVP's which could be used in pharmaceutical applications (Liu, Fullwood et al. 2000; Liu and Rimmer 2002b; D'Souza, Schowen et al. 2004). Liu et al. synthesised a number of allyloxycarbonyloxymethyl-5-fluorouracil monomers that were subsequently successfully reacted with NVP producing polymers. These were slightly rich in NVP at low conversions and rich in 5- fluorouracil (5-FU) monomer at the latter stages of the reaction and a reactivity ratio of $r_{NVP} = 0.97$ was reported (Liu, Fullwood et al. 2000). Subsequent studies involved these 5-FU functionalised NVP oligomers being used in the synthesis of NVP membranes. The release of the 5-FU from these membranes was highly non-Fickian with the release mechanism dominated by chemical degradation of carbonate groups present in the polymers (Liu and Rimmer 2002b). Cytotoxicity testing of these NVP/5-FU membranes showed that wells containing polymer with the 5-FU functionality were not viable. It was also apparent that cell proliferation but not viability was reduced in the presence of the polymers lacking the 5-FU group.

Functional NVP oligomers have also been prepared. These oligoNVP's with methyl ketone end groups could be subsequently transformed to produce oligoNVP's with silyl enol ether end groups (Liu and Rimmer 2002a). It has been suggested that living polymerisation methods such as Reversible Addition Fragmentation Chain

Transfer (RAFT) polymerisation could be used to produce NVP oligomers with functional end groups and narrow polydispersities (Moad, Mayadunne et al. 2003).

1.5.3 Hydrogels as wound dressings.

Wound dressings as described in section 1.4 have to meet a formidable list of criteria to be successful. Briefly they have to be tissue compatible, non-toxic, impermeable to exogenous microorganisms, have a rapid and sustained adherence to the wound surface, conform to surface irregularities, be elastic to permit motion of underlying tissue, have an appropriate tensile strength to resist fragmentation, have a long shelf life, minimal storage requirements, be low cost, minimise nursing care of wounds, minimise patient discomfort, be translucent to allow direct observation of healing, reduce healing time, and prevent heat and fluid loss (Seal, Otero et al. 2001; Sheridan and Tompkins 2002; Atiyeh, Hayek et al. 2005; Ehrenreich and Ruszczak 2006). Polymers used in hydrogel wound dressings include poly(ethylene oxide), polyacrylamides, PNVP, chitosan, glycosaminoglycans, pluronic F127 (a block copolymer of poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO)), PHEMA and PEG (Corkhill, Hamilton et al. 1989; Ramos-E-Silva and Ribeiro De Castro 2002)

Hydrogel dressings are available as either gels, sheets or impregnated gauze (Lionelli and Lawrence 2003). There are many advantages to gel dressings, they are soothing, they will follow the contours of the wound surface, are non-adherent, are semi-transparent, are relatively easy to apply and remove, are permeable to oxygen and water, and antimicrobial agents can be incorporated within the material (Quinn, Courtney et al. 1985; Eaglstein 2001; Menaker 2001). This last point is especially useful as hydrogels are poor bacterial barriers. Therefore an antimicrobial agent can be used in conjunction with the hydrogel dressing, either a cream underneath the dressing or an antimicrobial agent such as penicillin or PNVP-I being incorporated into the dressing (Corkhill, Hamilton et al. 1989; Cho and Lo 1998). Hydrogels have an ability to cool the skin upon application. This cooling is a result of their high specific heat and this has been quantified at about $5^{\circ}C$. The ability of hydrogels to cool the skin upon application reduces pain and inflammation (Cho and Lo 1998; Ramos-E-Silva and Ribeiro De Castro 2002). Hydrogels, unlike alginates or

hydrocolloids do not need further wound secretions to attain a gelatinous consistency. Hydrogels as well as donating moisture to the wound are capable of absorbing surplus contaminated exudates and safely retaining them within the gel structure. The absorption of secretions causes an expansion of the crosslinks in the polymer chains making room for the inclusion of foreign bodies such as bacteria, detritus, and odour molecules that are irreversibly taken up with the liquid (Jones and Vaughan 2005). One of the major problems with hydrogels is their relatively poor mechanical properties. The underlying cause of the limited resistance to mechanical deformation and poor tear strength of hydrogels is the plasticizing effect of the water held within the polymer network, Ironically, it is this same feature that dominated the surface properties, permselectivity and permeability that give hydrogels their unique and interesting properties (Corkhill, Hamilton et al. 1989). The poor material properties mean that hydrogels tend to be used as part of a composite dressing or with a secondary dressing. Other disadvantages include the need for frequent dressing changes and the fact that hydrogels do not provide a very good barrier against bacteria and actually selectively permit the overgrowth of gram-negative organisms. Also as hydrogel dressings are non-adherent bacteria and other pathogens may gain entry to the wound from the edges of the dressing (Cho and Lo 1998; Ramos-E-Silva and Ribeiro De Castro 2002). Hence the need for antimicrobial agents mentioned earlier.

Hydrogels are considered to be a standard form of management for sloughy or necrotic wounds. However, they are not recommended for wounds producing high levels of exudate or where there is evidence of gangrenous tissue. In this case the wound should be kept dry to reduce the risk of infection (Jones, Grey et al. 2006). They are also useful for painful wounds, post-dermabrasion, laser wounds, ulcers, chemical peels and partial thickness wounds, burns and scalds. Hydrogel dressings are also useful for rehydrating eschar and slough for easy removal from the wound surface. They are also useful for creating and maintaining a moist but not wet microenvironment over the healing wound (Eaglstein 2001; Ramos-E-Silva and Ribeiro De Castro 2002; Jones and Vaughan 2005).

Examples of hydrogel dressings include Aquaform, Carrasyn, Curafil, Curagel, Flexderm, FlexiGel, Geliperm, GranuGel, Hydron Burn Bandage, Intrasite, Lamin,

Nu-Gel, Omniderm, Purilon, Skintegrity Hydrogel, SoloSite, Sterigel, Tegagel, and Vigilon (Quinn, Courtney et al. 1985; Corkhill, Hamilton et al. 1989; Eaglstein 2001; Lionelli and Lawrence 2003; Jones and Vaughan 2005; Jones, Grey et al. 2006; Ovington 2007). Of these dressings Geliperm (an agar and polyacrylamide gel) and Vigilon (a poly(ethylene oxide) hydrogel with a polyethylene backing film) have shown promise in the treatment of burns (Quinn, Courtney et al. 1985).

1.5.4 Hydrogels in drug delivery applications.

Hydrogels are commonly used vehicles for drug delivery applications. Drugs can range from small molecules such as 5-fluorouracil (5-FU), 4-methyl umbelliferone (4-MU) or β-aminopropionitrile fumarate (βAPN) to larger molecules such as proteins, enzymes and DNA or RNA. According to Saltzman (2001) many decades after the first descriptions of biocompatible polymers for drug delivery, a handful of these are now approved for use in humans (Saltzman 2001). The selection of hydrogels used in pharmaceutical applications depends on the characteristics of the gel and on the application of the drug or protein. Hydrogels have several important characteristics involved in drug diffusion. These include the ionisation of the gel, the swelling ratio, and specific mesh or pore size. Functional groups along the polymer chain can also react to the external environment for example temperature, pH, ionic strength of the swelling agent or a combination of two or more factors (Devine, Devery et al. 2006).

Drug release from polymeric materials has been extensively investigated in the literature. Model drugs varying from large proteins such as bovine serum albumen (BSA) (Anseth, Metters et al. 2002), lysozyme and IgG (Hennink, Talsma et al. 1996) to small molecules such as 5-fluorouracil (Liu and Rimmer 2002b) have been used in these studies. The group of Peppas et al. studied the kinetics of polymer-solvent-solute interactions extensively (Ritger and Peppas 1987a; Ritger and Peppas 1987b; Brannon-Peppas and Peppas 1991; Brazel and Peppas 2000). Ritger and Peppas (1987a; 1987b) attempted to simplify the data analysis involved in the application of the existing Fickian diffusion equation, equation 1.1 which deals with the kinetics of drug release from polymers.

$$\frac{M_t}{M_\infty} = 1 - \sum_{n=0}^{\infty} \frac{8}{(2n+1)^2 \pi^2} \exp\left[\frac{-D(2n+1)^2 \pi^2}{l^2} t\right]$$
<div style="text-align: right;">Equation 1.1</div>

M_t = Mass of drug released at time, t

M_∞ = Mass of drug released as time approaches infinity

n = Diffusional exponent

D = Drug diffusion coefficient

l = sample thickness

The fractional release (M_t/M_∞) of the drug from polymers with varying geometries relates to the release time (t) using the proposed empirical, exponential expression $M_t/M_\infty = kt^n$ where n = diffusional exponent and k = rate constant. Using this equation generally "Fickian diffusion is defined by *n* equal to 0.50 and non-Fickian by n greater than 0.50" (Ritger and Peppas 1987a). Fickian diffusion is defined as zero order release of the solute from the polymer i.e. steady state diffusion, no interactions between the solute and the polymer. Non-Fickian diffusion is indicative of interactions between the solute and the polymer. Sample geometry influences solute release. Ritger et al. (1987b) showed how the diffusion exponent varies for samples with different geometries in swellable systems. For cylindrical samples, such as the ones used in this study, Ritger states that for Fickian diffusion n=0.45, for anomalous (non-Fickian) transport n= 0.45<n<0.89 and for Case-II transport n=0.89. Whilst Case-I or Fickian diffusion is described by a diffusion exponent, Case-II transport is described by a characteristic relaxation constant. Brazel and Peppas (2000) used mathematical models to predict drug release profiles from polymers. These predictions showed that sample thickness, water and drug diffusion exponents and polymer viscoelastic response markedly change the release profiles. A small change in sample thickness can have a large effect on drug release behaviour.

The controlled release of proteins from hydrogels has been extensively investigated. The release of model proteins such as lysozyme, bovine serum albumin (BSA) and immunoglobulin G (IgG) has been investigated from dextran based hydrogels (Hennink, Talsma et al. 1996; Meyvis, De Smedt et al. 2001). BSA has also been used as the model protein in release experiments from glycidyl methacrylate-

hyaluronic acid and glycidyl methacrylate-hyaluronic acid PEG hydrogels (Leach, Bivens et al. 2003). In this study the protein was also loaded into the hydrogels in two forms. The BSA was mixed into the hydrogel monomer blend before crosslinking or loaded into PLGA microspheres which were mixed into the monomer blend before crosslinking. The presence of the microspheres was shown to extend the release of the BSA from the system from a few hours to a couple of weeks (Leach and Schmidt 2005). The use of microspheres of different types i.e. liposomes, hydrosomes, polymersomes are becoming more and more popular for drug delivery, especially for the delivery of fragile molecules such as proteins. Bos et al. (2001) report the encapsulation of a wide variety of proteins into a variety of synthetic, i.e. PLGA, dextran methacrylate or dextran HEMA, microspheres which were subsequently incorporated into the PEGT/PBT copolymer polyActive and various hydrogels. These systems proved to be extremely versatile with systems for both prolonged and delayed release patterns being achieved (Bos, Verrijk et al. 2001).

Crosslinked nanoparticles synthesised from PNVP have been designed for use as a vector for gene delivery. According to Saxena et al. (2006) it has been observed that PNVP uptake by cells and its effect on the intracellular enzymes was very limited. Moreover, PNVP is retained within the intracellular vesicles after its uptake. The PNVP nanoparticles synthesised in this study were able to evade the rough endoplasmic reticulum and therefore were able to remain in circulation for a considerable period of time and resulted in an 80% transfection efficiency (Saxena, Mozumdar et al. 2006). Drugs like 5-fluorouracil (5-FU) can be conjugated to allyl carbonate crosslinkers and then used to produce 5-FU releasing PNVP hydrogels (Liu and Rimmer 2002b). The 5-FU was released from these hydrogels by degradation of the carbonate groups. The release rates of the 5-FU ($37^{\circ}C$ and pH 7.4) from the PNVP hydrogels investigated in this study showed that the release is controlled by Fickian diffusion and interaction with the polymer chains with the expected contribution from the rate of crosslinker degradation. The diffusion exponents obtained for the release of 5-FU from PNVP hydrogels were between 0.13 and 0.23 and increased with increasing amounts of the 5-FU functionalised crosslinker (Liu and Rimmer 2002b). Alternatively D'Souza et al. (2004) conjugated the drug *para*-nitroaniline (PNA) directly to the PNVP chain by opening the lactam ring and protection of the resulting amine with t-BOC prior to conjugation of the

opened ring with the PNA. The t-BOC could then be removed to produce a secondary amine. In the deprotected polymers approximately 35% of the PNA was released over a 30 day period (50°C and pH 7.5). The protected polymers released only 12% of the PNA in the same conditions and over the same time period. D'Souza et al. suggest that these PNVP-PNA conjugates could be suitable for controlled release applications due to the relatively slow release kinetics. Kaneda et al. (2004) successfully conjugated TNFα to PNVP via a reaction with methoxypolyethylene glycol-succinimidyl succinate. PNVP-TNFα after i.v. injection has a much longer retention time in the blood than native TNFα with a half life of 360.1 min ± 45.7 min opposed to 4.6 min ± 2.2 min for native TNFα. PEG-TNFα was also synthesised and this had a half life 122.6 min ± 85.0 min. PNVP-TNFα was shown to have a more potent antitumor effect than the PEG-TNFα. These polymers also showed little sign of tissue accumulation.

Shantha and Harding (2003) reported entrapment of propranolol hydrochloride (PPH) into poly(lactose acrylate-N-vinyl-2-pyrrolidinone) hydrogels. Release of PPH from the PNVP based hydrogels followed a characteristic release pattern over a period of 48 hours. There was a slight burst release during the first hour of the experiment explained by PPH adsorbed onto the hydrogel surface. Approximately 54% of the entrapped drug was released by hour six. During this initial six hour period the release profile followed a near zero-order pattern. This is indicative of drug release via diffusion from the hydrogel matrix as it swells to equilibrium. PNVP has been copolymerised with polyacrylic acid and aspirin and paracetamol incorporated (Devine, Devery et al. 2006). NVP has also been copolymerised with itaconic acid and methylene blue incorporated as a model drug in this system (Sen and Guven 1999). Copolymerisation of PNVP with acrylic acid allowed for the incorporation of vitamin B_{12} (Bajpai and Dubey 2005). These pH sensitive hydrogels showed maximum release at pH 6.8 with a minimum release at pH 1.2.

1.6 Aims.

In summary whilst great strides have been taken in understanding wound healing and in developing wound dressings to aid healing, skin graft contracture is still a major problem facing burns patients. Recent work by others in the group had identified two candidate drugs for blocking skin contraction in tissue engineered skin. It was therefore proposed to develop a wound dressing based on the polymer PNVP that could be used to deliver the drugs and prevent or at the very least reduce skin graft contracture in burns patients. To develop this active hydrogel dressing a series of specific objectives were identified.

1. To evaluate the cellular cytotoxicity of novel PNVP hydrogels.

2. To examine the cytotoxicity of the drugs β-aminopropionitrile and 4-methyl umbelliferone.

3. To determine whether β-aminopropionitrile and 4-methyl umbelliferone would prevent or reduce contracture in a collagen gel contraction model. (The β–aminopropionitrile had been previously identified on the basis of its action on a 3D skin contraction model in this group and the 4-methyl umbelliferone on the basis of its action on fibroblast populated collagen gels in the group of M Edwards at the University of Glasgow).

4. To determine if 4-methyl umbelliferone could prevent or reduce contracture in the reconstructed skin contraction model.

5. To examine the loading and release of β-aminopropionitrile and 4-methyl umbelliferone into and from PNVP hydrogels.

6. To examine to what extent hydrogels loaded with β-aminopropionitrile and 4-methyl umbelliferone would prevent or reduce the contraction of the collagen gel contraction model and the 3D reconstructed skin model.

2. Materials and Methods.

2.1 Polymer synthesis and characterisation.

Materials.

1-vinyl-2-pyrrolidinone (NVP), Ethylene glycol dimethacrylate (EGDMA), Acrylic Acid (AA) and Dimethyl sulfoxide (DMSO) were obtained from Aldrich, UK. Absolute Alcohol, Acetone, Ether, Dimethylformamide (DMF), Tetrahydrofuran (THF), Propan-2-ol (IPA), Dichloromethane (DCM) and Acetonitrile were obtained from Fisher UK. Diethylene glycol bisallylcarbonate (DEGBAC) was obtained from Greyhound chromatography, UK. 2,2'-Azobis(2-methylpropionitrile) (AIBN) was obtained from Fluka, UK. 100 μm poly(ethylene terephthalate) (PET) sheeting was obtained from HiFi Industrial Film Ltd. UK), Benzene Dithiobenzoate (BDTB) was synthesised by Dr Steve Carter, Chemistry dept, University of Sheffield, UK synthesis unpublished, 1-methoxyphenyl-1-(trimethylsilyloxy)ethylene (MPTMSE) was synthesised by Dr Prodip Sarker, Chemistry dept, University of Sheffield, UK (Lang, Sarker et al. 2004).

2.1.1 Synthesis of poly(vinylpyrrolidinone) oligomers.

3 g distilled NVP was placed in a dried degassed flask with 30 mg AIBN. The reaction was performed at 60°C and slowly stirred for 5 minutes and then quenched with liquid nitrogen. The resulting foam was dissolved in dichloromethane and added slowly to gently stirring ether, the poly(vinylpyrrolidinone) oligomers precipitate out. The solution was filtered and the solid placed in a drying oven at room temperature overnight.

2.1.2 Synthesis of benzene dithiobenzoate functionalised poly(vinylpyrrolidinone) oligomers.

3 g distilled NVP was placed in a dried degassed flask with 0.464 g benzene dithiobenzoate and 30 mg AIBN. The reaction was performed at 60°C for 2 hours and then quenched with liquid nitrogen. The resulting oil was added slowly to gently

stirring ether, the poly(vinylpyrrolidinone) oligomers precipitate out. The solution was filtered and the solid placed in a drying oven at room temperature overnight.

2.1.3 Synthesis of 1-methoxyphenyl-1-(trimethylsilyloxy)ethylene functionalised poly(vinylpyrrolidinone) oligomers.

0.36 ml distilled NVP was placed in a dried degassed flask with 0.16 ml 1-methoxyphenyl-1-(trimethylsilyloxy)ethylene and 65 mg AIBN. The reaction was performed at 60°C for 2 hours and then quenched with liquid nitrogen. The resulting oil was added slowly to gently stirring ether, the poly(vinylpyrrolidinone) oligomers precipitate out. The solution was filtered and the solid placed in a drying oven at room temperature overnight.

2.1.4 Synthesis of poly(vinylpyrrolidinone) membranes in the presence of solvent.

10 g distilled NVP, 0.1 g of EGDMA, and 0.1 g of AIBN were placed into a beaker containing 4.4 g DMSO. The solution was gently stirred whilst dry nitrogen was bubbled through for 20 minutes. The monomer blend was then injected into a glass mould consisting of two glass tiles lined with 100 μm PET sheets sandwiching a PTFE 1 mm thick spacer. The volume of the mould was approximately 10 ml. The mould was then placed into an oven at 60°C for 24 hours. After 24 hours the glassy plaque was removed from the mould and placed into absolute alcohol. The alcohol was changed every 24 hours for 5 days.

2.1.5 Synthesis of poly(vinylpyrrolidinone) membranes in the absence of solvent.

Table 2.1 Formulations of monomer blends for the production of NVP hydrogels with different crosslinker concentrations in the absence of solvent.

Weight crosslinker	NVP / g	Crosslinker / g	AIBN / g
1 wt%	14.85	0.15	0.1
2 wt%	14.7	0.3	0.1

3 wt%	14.55	0.45	0.1
4 wt%	14.4	0.6	0.1
1 wt% + 1 wt% AA	14.7	0.15 + 0.15 AA	0.1

Distilled NVP, crosslinker(s) (1 – 4 wt%), and AIBN, in quantities detailed in table 2.1 above, were placed into a beaker. The solution was gently stirred whilst dry nitrogen was bubbled through for 20 minutes. The monomer blend was then injected into a glass mould consisting of two glass tiles lined with 100 μm PET sheets sandwiching a PTFE 1mm thick spacer. The mould was then placed into an oven at 60°C for 24 hours. After 24 hours the glassy plaque was removed from the mould and placed into absolute alcohol. The alcohol was changed every 24 hours for 5 days.

2.1.6 Synthesis of poly(vinylpyrrolidinone) membranes incorporating 3-aminopropionitrile fumarate.

Table 2.2 Formulations of monomer blends for the production of NVP hydrogels containing 3-aminopropionitrile fumarate with different crosslinker concentrations in the presence or absence of water.

Weight crosslinker	NVP / g	Crosslinker / g	AIBN / g	βAPN.F / mg	Water
1 wt%	14.85	0.15	0.1	3, 3.5, 10.5	0, 1 ml
1 wt% + 20 wt% H_2O	11.85	0.15	0.1	3, 3.5, 10.5	3 g
2 wt%	14.7	0.3	0.1	3, 3.5, 10.5	0, 1 ml
3 wt%	14.55	0.45	0.1	3, 3.5, 10.5	0, 1 ml
4 wt%	14.4	0.6	0.1	3, 3.5, 10.5	0, 1 ml
1 wt% + 1 wt% AA	14.7	0.15 + 0.15 AA	0.1	3, 3.5, 10.5	N/A

Distilled NVP, crosslinker(s), AIBN and water, in quantities detailed in table 2.2 above, were placed into a beaker containing 3 mg, 3.5 mg or 10.5 mg of 3-aminopropionitrile fumarate. The solution was gently stirred whilst dry nitrogen was bubbled through for 20 minutes. The monomer blend was then injected into a glass mould consisting of two glass tiles lined with 100 μm PET sheets sandwiching a PTFE 1 mm thick spacer. The mould was then placed into an oven at 60°C for 24 hours. After 24 hours the glassy plaque was removed from the mould.

2.1.7 Production of poly(vinylpyrrolidinone) xerogels.

Alcohol swollen P(NVP-co-EGDMA) and P(NVP-co-DEGBAC) membranes were cut to size with a cork borer in sterile conditions. The hydrogel discs were placed into a Petri-dish and the lid was fastened with autoclave tape. The Petri-dish was then placed into a drying oven at 50°C for 48 hours. The xerogels thus formed were then stored in sterile containers at room temperature until needed.

2.1.8 Equilibrium water content measurements.

Phosphate buffered saline (PBS) swollen hydrogel membranes were stored at room temperature prior to measurements. The swollen membranes were cut with a size 3 cork borer (diameter 7 mm). The excess solvent was gently removed from the hydrogels by gently dabbing the polymer with tissue. The polymer pieces were weighed and placed on a glass slide. The slide was placed into a vacuum oven at 50°C for 24 hours. The polymer pieces were weighed again and the equilibrium water content calculated using the formula.

$$EWC = \frac{\text{wet weight - dry weight}}{\text{wet weight}} \times 100$$

2.1.9 ^{1}H NMR.

Approximately 5 mg of the sample was dissolved in 1 ml of the appropriate deuterated solvent. The ^{1}H NMR spectra were obtained using a Bruker AC250 spectrometer at 250MHz and analysed using Spinworks1.3 software.

2.1.10 ^{13}C solid state NMR.

^{13}C solid state NMR analysis was kindly performed by Dr David Apperley at the University of Durham EPSRC solid state NMR service. A Varian Unity Inova Spectrometer with a 7.5 mm MAS probe was used. Approximately 500 mg of xerogel was sent to the University of Durham by first class post. At The University of Durham the samples were lightly ground in a mortar and pestle prior to analysis.

2.1.11 Size exclusion chromatography (SEC).

SEC was performed using 2 x 40 cm kiw molecular weight Styrogel^{TM} 5 mm mixed gel columns at room temperature. The eluent was THF at a flow rate of 1.0 cm^3.min^{-1}. Calibration was performed using polystyrene standards and a combination of RI and UV detection was employed. RI detector was a ERC-7512 obtained from ERMA inc. and the UV detector was a Lambda-Max LC spectrophotometer model 481. Automated sampling used a Gilson auto injector. All detectors were attached to a PC running Cirrus^{TM} GPC software.

2.1.12 Compression testing.

Compression testing was performed on a BOSE electroforce 3200 attached to a PC running WinTest^{TM} software. PBS swollen hydrogel discs of diameter 13.2 mm were cut with a cork borer. Compression testing was performed over a distance of 2 mm at a rate of 0.1 mm.s^{-1}.

2.1.13 Degradation studies.

PNVP samples hydrated in PBS were placed in a sealed container with a known quantity of PBS. The container was then placed in an incubator at 37°C and samples were removed and EWCs calculated for up to 12 days.

2.2 Cell Culture.

Materials.

Dulbeccos' Modified Eagles Medium (DMEM) and Hams F12 were obtained from Sigma, UK or Biowest Biosera. Foetal calf serum was obtained from Biowest Biosera. L-glutamine, amphotercin B, penicillin-streptomycin, human recombinant epidermal growth factor, hydrocortisone, cholera toxin, adenine, insulin, apo-transferrin 3,3,5-tri-idothyronine (TT), trypsin-ethylene diamine tetra-acetic acid (EDTA) (0.05% w/v trypsin/ 0.02% w/v EDTA), 0.02% w/v EDTA, Collagenase A, 0.4% w/v Trypan blue, Bovine Serum Albumen (BSA) and Fibronectin were obtained from Sigma, UK. Epilife medium and human corneal growth supplement were obtained from Cascade Biologics, UK. Phosphate buffered saline tablets (Dulbeccos' A) were obtained from Oxoid, UK. Collagen from rats tail was obtained from Fluka, UK. Trypsin 0.1% w/v was obtained from Difco, UK. Mycoplasma removal agent (MRA) was obtained from Serotec, UK.

Skin for cell isolation and production of de-epithelised acellular dermis (DED) was obtained from patients undergoing routine abdominoplasties and breast reductions at the Northern General Hospital, Sheffield. All patients gave full informed consent for skin to be used for research through a protocol approved by the Ethical Committee of the Northern General Hospital Trust, Sheffield, UK.

Adenine 0.5 g of powder was dissolved in 20 ml of distilled water. 1 M HCl was added drop-wise to get the powder into solution and the volume made up to 80 ml with distilled water before being filter sterilised, 2 ml aliquots were stored at -20°C before use. Final concentration 1.85×10^{-4} M, 2 ml aliquot used in making up 500 ml medium.

Cells The J2 clone of the NIH 3T3 strain of murine fibroblasts was donated by Professor Howard Green at the Massachusetts Institute of Technology, USA or isolated from split thickness skin as per section 2.2.6 and section 2.2.7.

Cholera Toxin 1 mg of cholera toxin was dissolved in 1.18 ml of distilled water and stored at 4°C. 0.01 ml of this solution was added to 1 ml of medium containing serum to form a base stock 8.47 $pg.ml^{-1}$, which was then stored at 4°C. 0.5 ml was used in making up 500 ml medium.

Collagenase A was prepared by dissolving Collagenase A powder in fibroblast culture medium in sterile conditions to make 0.05% (w/v) solution.

'Difco Trypsin' 0.1% w/v was prepared by the adding of 0.5 g of Difco Trypsin powder, 0.5 g D-glucose, and 0.5 ml phenol red to 500 ml PBS. This was adjusted to pH 7.45 using 2 M NaOH using a pH meter. This was filter sterilised and aliquots stored at -20°C until needed.

Epidermal Growth Factor (Human Recombinant from E.coli) 0.1 mg of EGF was dissolved in 1 ml of 10% Greens medium. 0.05 ml aliquots were prepared and stored at -20°C until needed. 0.05 ml used in making up 500 ml Greens medium, final concentration 10 ng.ml^{-1}.

Hydrocortisone 250 mg was dissolved in 1 ml 100% ethanol. 1 ml of this was dissolved in 9 ml of PBS to form a base stock and stored at 4°C. 0.08 ml of stock used in making up 500 ml medium.

Insulin 0.01 g was dissolved in 1 ml of 0.01 M HCl and then added to 9 ml of distilled water. This was filter sterilised and stored at 4°C. 0.5 ml of stock used in making up 500 ml medium.

PBS was prepared by adding 1 tablet per 100 ml of distilled water. This solution was autoclaved at 115°C for 15 minutes and left to cool before use.

Transferrin/T3 13.6 mg T_3 was dissolved in a minimum amount of 0.02 M NaOH and the volume made up to 100 ml. 250 mg apo-transferrin was dissolved in 30 ml PBS, 0.5 ml T_3 added and volume made up to 50 ml. This was filter sterilised and 0.5 ml aliquots stored at -20°. Final concentration T_3 1.36 μgl^{-1}, transferrin 2.5 mg.l^{-1} 0.5 ml used in making up 500 ml medium.

Trypan Blue stock solution 1:1 mixture of 0.4% Trypan blue and PBS

No. 22 scalpel blades were purchased from Swann-Morton, Sheffield, UK. Cell counts were performed using a modified Neubauer haemocytometer purchased from Weber Scientific International, UK. Stainless steel rings and grids were manufactured by the Department of Medical Physics at the Royal Hallamshire Hospital, Sheffield, UK. Plasticware for cell culture was obtained from Costar, UK. ThinCert™ cell culture inserts were purchased from Greiner, UK. All cells, tissues and sterile reagents were handled in Class II laminar flow hoods obtained from Walker Safety Cabinets, UK. Cell and tissue culture was performed at 37°C, 5% CO_2

95% humidity, in Sanyo CO_2 incubators. Light microscopy of cell cultures was performed using an Olympus CK40-F200 light and phase contrast microscope. Still photographs were taken using a Nikon Coolpix 990 digital camera.

2.2.1 Fibroblast culture medium.

DMEM high glucose (4500 mg.l^{-1} glucose) supplemented with 10% v/v foetal calf serum (FCS), 2 x 10^{-3} M l-glutamine, 0.625 μg.ml^{-1} amphotercin B, 100 I.U.ml^{-1} penicillin and 100 μg.ml^{-1} streptomycin.

The above ingredients were mixed at room temperature in a class II laminar flow hood to make a total volume of 500 ml. Medium was stored at <4°C for a maximum of 6 weeks prior to use. An aliquot of medium was warmed to 37°C before use.

2.2.2 Serum free fibroblast culture medium.

DMEM high glucose (4500 mg.l^{-1} glucose) supplemented with 2 x 10^{-3} M l-glutamine, 0.625 μg.ml^{-1} amphotercin B, 100 I.U.ml^{-1} penicillin and 100 μg.ml^{-1} streptomycin.

The above ingredients were mixed at room temperature in a class II laminar flow hood to make a total volume of 500 ml. Medium was stored at <4°C for a maximum of 6 weeks prior to use. An aliquot of medium was warmed to 37°C before use.

2.2.3 3T3 culture medium.

DMEM high glucose (4500 mg.l^{-1} glucose) supplemented with 10% v/v new born calf serum (FCS), 2 x 10^{-3} M l-glutamine, 0.625 μg.ml^{-1} amphotercin B, 100 I.U.ml^{-1} penicillin and 100 μg.ml^{-1} streptomycin.

The above ingredients were mixed at room temperature in a class II laminar flow hood to make a total volume of 500 ml. Medium was stored at <4°C for a maximum of 6 weeks prior to use. An aliquot of medium was warmed to 37°C before use.

2.2.4 Greens medium.

A clinically approved cell culture medium suitable for the culture of keratinocytes. However fibroblasts are also able to grow successfully in Greens medium (Sun, Mai et al. 2005).

DMEM high glucose and Ham's F12 medium in a 3:1 ratio supplemented with 10% v/v foetal calf serum (FCS), 10 ng.ml^{-1} human recombinant epidermal growth factor, 0.4 μg.ml^{-1} hydrocortisone, 10^{-10} M cholera toxin, 1.8 x 10^{-4} M adenine, 5 mg.ml^{-1} insulin, 5 μg.ml^{-1} apo-transferrin, 2 x 10^{-7} M 3,3,5-tri-idothyronine, 2 x 10^{-3} M glutamine, 0.625 μg.ml^{-1} amphotercin B, 100 I.U.ml^{-1} penicillin and 100 μg.ml^{-1} streptomycin.

The above ingredients were mixed at room temperature in a class II laminar flow hood to make a total volume of 500ml. Medium was stored at <4°C for a maximum of 6 weeks prior to use. An aliquot of medium was warmed to 37°C before use.

2.2.5 HCEC medium.

Epilife was supplemented with Human Cornea growth supplement.

2.2.6 Cell counts and viability assessment.

Cell suspension, Trypan blue solution, Cell culture media.

Cell counts and viability assessments were performed using trypan blue. Cells were suspended in a known volume of cell culture media. 20 μl of this cell suspension was removed and 20 μl of Trypan blue stock solution was added. Non viable cells appear blue. Viable cells were counted using a Neubauer haemocytometer and the concentration of viable cells in suspension was calculated.

2.2.7 Isolation and culture of keratinocytes from split thickness skin grafts.

Split thickness skin from theatre (no more than 3 days old), PBS, 'Difco-Trypsin', Foetal calf serum, Greens medium, Trypan blue solution, 0.02% w/v EDTA solution, Trypsin/EDTA solution

Keratinocyte isolation, split thickness skin (STS) (approximately 2.5 cm^2) was cut into thin pieces (approximately 0.5 cm x 1 cm) and placed into 10 ml 'Difco-Trypsin'. This solution was incubated at 4°C overnight (typically 12-18 hours). Enzymatic activity was stopped after this time by the addition of 5 ml foetal calf serum. The skin strips were gently placed into a Petri dish containing a small amount (approximately 1 ml) of PBS. The epidermis was gently peeled off the dermis and the bottom of the epidermis and top of the dermis gently scraped to remove cells (keratinocytes, melanocytes etc). The PBS and cells were transferred to a universal containing a 50/50 mix PBS and FCS. Cells in PBS and FCS were spun down at 1000 rpm for 5 minutes and then suspended in 10% Greens medium. A cell count was performed using trypan blue to highlight non viable cells. Cells were seeded at a density of $\approx$ 4 x 10^6 in T75 flasks seeded approximately 1 hour earlier with 1 x 10^6 i3T3. Cells were cultured at 37°C, 5% CO$_2$ in a humidified atmosphere. The media was changed after 24 hours and subsequent media changes were performed every 2-3 days and the desired 70-80% confluency was generally achieved in 5-7 days.

Keratinocyte sub-culture, the media was rinsed away with PBS and the i3T3 cells were removed by incubation with 5 ml 0.02% w/v EDTA at 37°C. The flasks were examined by phase contrast microscopy every 5 minutes and gently tapped to encourage fibroblast detachment ensuring that the keratinocytes were still attached. The i3T3 containing EDTA solution was then removed and the flask rinsed with PBS. 2.5 ml Trypsin/EDTA was then added to the flask and incubated at 37°C. Keratinocyte detachment was encouraged by gentle tapping and confirmed by phase microscopy after 5 minutes. The cell suspension was added to 10 ml 10% Greens medium to neutralise the trypsin and spun down at 1000 rpm for 5 minutes. The resulting pellet was then suspended in a known volume of 10% Greens medium and a cell count was performed prior to use, again using trypan blue. Keratinocytes were used between passages 1 and 3. When keratinocytes were cultured without i3T3's the TCP was coated with 0.2 mg.ml^{-1} collagen I solution.

2.2.8 Isolation and culture of fibroblasts from split thickness skin grafts.

Split thickness skin from theatre (no more than 3 days old), PBS, Collagenase A, Foetal calf serum, Fibroblast culture medium, Mycoplasma Removal Agent.

Fibroblast isolation, the dermis left from keratinocyte isolation is minced up and placed into a Petri dish containing collagenase A solution and incubated at $37^\circ C$ overnight. 10 ml of fibroblast culture medium is added to the Petri dish to stop collagenase. The cell solution was removed spun down at 2000 rpm for 10 minutes. The cells were then suspended in fibroblast culture medium containing mycoplasma removal agent and seeded in a T25 flask and incubated at $37^\circ C$, 5% CO_2 in a humidified atmosphere. The medium was changed after 24 hours then every 3-4 days until the cells were 80% confluent.

Fibroblast sub-culture, 2 ml Trypsin/EDTA was added to the fibroblast containing flasks after thorough washing with PBS and incubated at $37^\circ C$. Fibroblasts were encouraged to detach by gentle tapping and detachment was confirmed by phase contrast microscopy. This generally took around 5 minutes. The cell suspension was added to 10 ml fibroblast culture medium to neutralise the trypsin, the cell suspension was then spun down at 1000 rpm for 5 minutes. The pellet was then suspended in a known volume of fibroblast culture medium and a cell count performed prior to use. Fibroblasts were used between passages 3 and 9.

2.2.9 Culture of HaCaT cells.

The HaCaT human keratinocyte cell line was kindly supplied by Professor N.E. Fusenig (Institute of Biochemistry, German Cancer Research Centre, Heildelberg, Germany) and cultured in fibroblast culture medium at $37^\circ C$, 5% CO_2 in a humidified atmosphere.

Sub-culture Flasks containing HaCaT cells were washed thoroughly with PBS and then were incubated with 5 ml 0.02% w/v EDTA at $37^\circ C$ for 5 minutes, 2 ml trypsin/EDTA was then added and cells were incubated for a further 5 minutes. HaCaT's were encouraged to detach by gentle tapping and detachment was confirmed by phase contrast microscopy. This generally took another 5 minutes. The

cell suspension was added to 10 ml fibroblast culture medium to neutralise the trypsin. The cell suspension was then spun down at 1000 rpm for 5 minutes. The pellet was then suspended in a known volume of fibroblast culture medium and a cell count performed prior to use.

2.2.10 Culture of Human Corneal Epithelial Cells (HCEC).

A HCEC line (10.014 pRSV-T) was obtained from ATCC, USA. HCEC's were cultured on tissue culture polystyrene (TCP) plates coated with a solution of Bovine Serum Albumin (0.01 mg.ml^{-1}), Fibronectin (0.01 mg.ml^{-1}) and Collagen 1 (0.03 mg.ml^{-1}), in HCEC medium. Cells were cultured in a humidified atmosphere at 37°C, 5% CO_2 and used between passages 45 and 47.

Sub-culture After washing thoroughly with PBS flasks of HCEC cells were incubated with 2 ml trypsin/EDTA at 37°C for approximately 2 minutes. HCEC's were encouraged to detach by gentle tapping and detachment was confirmed by phase contrast microscopy. The cell suspension was added to 5 ml trypsin inhibitor and then spun down at 1000 rpm for 5 minutes. The pellet was then suspended in a known volume of fibroblast culture medium and a cell count performed prior to use.

2.2.11 3T3 murine fibroblast culture and irradiation.

Irradiated 3T3 (i3T3) murine fibroblasts were used as a feeder layer during keratinocyte culture. A known number of proliferative 3T3's were stored at passage 14 in cryovials containing 1 ml of cryopreservation medium (see section 2.3.1) in liquid nitrogen (-196°C).

For production of i3T3 passage 14 3T3's were thawed and expanded using standard fibroblast sub-culture protocol (Section 2.2.8). Once sufficient cell numbers had been achieved (this was usually achieved at passage 17) the cells were then sub-cultured again. However once in suspension the cells were irradiated by exposure to a cobalt-60 source. Cells of known concentration in 3T3 culture medium were placed into 25 ml Universal containers. They were then exposed to γ-irradiation and received a total radiation dose of 25 Grays.

2.3 Cell cryopreservation.

Materials.

Foetal Calf Serum, (DMSO), Cellstar Cryovials were obtained from Greiner Bio-one, UK, Nalgene™ Cryo 1°C freezing container obtained from Nalgene Co., USA.

2.3.1 Cryopreservation medium.

1 ml dimethyl sulphoxide (DMSO) was added to 9 ml foetal calf serum to produce a 10% solution of DMSO in FCS. This solution was made up fresh each time it was needed.

2.3.2 Method of cryopreservation.

Immediately prior to cryopreservation, the cells were detached from their culture flasks using trypsin/EDTA and suspended in a known volume of the appropriate culture medium. A cell count was performed and the cells were spun down at 1000 rpm for 5 minutes. The cells were then suspended in cryopreservation medium to give a total cell count of 1-4 x 10^6 cells per ml depending on the cell type. The cryovials were placed in a Nalgene Freezing Container and placed into a -80°C freezer overnight. The cryovials, now containing frozen cells were then transferred to a Dewar bucket containing liquid nitrogen (-196°C).

2.3.3 Thawing of cryopreserved cells.

Cryovials were removed from the liquid nitrogen and defrosted in a water bath at 37°C for few minutes. They were then added to 10 ml pre-warmed cell culture medium. The cells were then spun down at 1000 rpm for 5 minutes. The supernatant was then discarded and the cell pellet suspended in a known volume of cell culture medium. A cell count and viability assessment was then performed.

2.4 Mono-and co-culture of keratinocytes and fibroblasts on tissue-culture plastic for drug cytotoxicity experiments.

Materials.

Costar[TM] 24-well tissue culture plates, were obtained from Corning, USA. 0.20 μm non-pyrogenic sterile filters were obtained from Sarstedt, Germany. Cells, Greens culture medium, fibroblast culture medium, 3-aminopropionitrile fumarate, 4-methyl umbelliferone sodium salt.

3-aminopropionitrile (βAPN) solution 3-aminopropionirile fumarate was dissolved in DMEM to produce a stock concentration of 10 mg.ml^{-1}. This was filter sterilised and stored at 4°C until needed (no more than 4 weeks).

4-Methyl umbelliferone (4-MU) solution 4-MU sodium salt was dissolved in DMEM to produce a stock concentration of 0.1 M. This solution was filter sterilised and made fresh for each experiment.

Keratinocytes were cultured as per section 2.2.7 and then plated into 24-well culture plates coated with 0.2 mg.ml^{-1} rat tail collagen at 5×10^4 cells in 1 ml Greens medium. Cells at passage 1 and 2 were used. Human skin fibroblasts were cultured as per section 2.2.8 and cells at passage numbers 3 to 8 were used. They were plated in fibroblast culture medium for monoculture experiments and Greens medium for co-culture experiments. Cells were plated in 24-well culture plates at 5×10^4 cells per well in 1 ml medium. For co-culture experiments, 1.25×10^4 fibroblasts and 3.75×10^4 keratinocytes were plated together in a total of 1 ml Greens medium. Experiments were performed over 7 days.

2.5 Culture of cells in direct and indirect contact with PNVP polymers.

Materials.

Costar™ 24-well tissue culture plates, Costar™ 12-well tissue culture plates, Greiner ThinCert™ cell culture inserts, stainless steel rings, cells, cell culture medium, PNVP polymers.
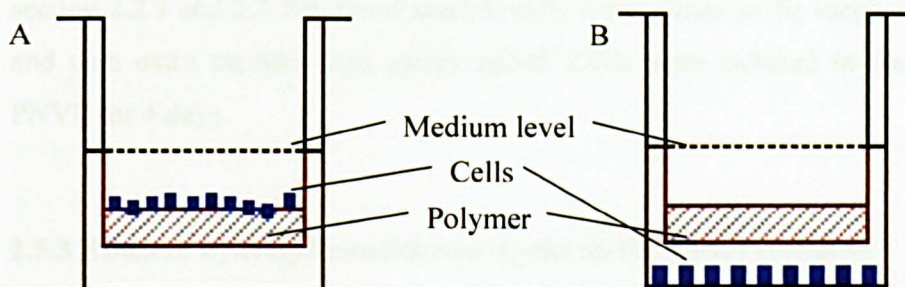
A — Medium level
Cells
Polymer

B

Figure 2.1 Cartoon depicting a) indirect contact culture system and b) direct contact culture system.

2.5.1 Culture of cells in direct contact with PNVP polymers.

A cartoon illustrating the experimental set up is shown in figure 2.1.A. Basically PNVP hydrogels were placed into ThinCert™ PET tissue culture inserts with 8 μm pores and suspended in 24 well plates. Cells were seeded at 5×10^4 cells per well in 10 μl of medium into the ThinCert on top of the hydrogel. This ensured contact between the cells and the PNVP. Alternatively to ensure contact between the cells and the hydrogels, hydrogel discs of 18 mm diameter were cut and placed into 12 well Costar™ plates. Stainless steel rings, internal diameter 10 mm were placed on top of the gels and cells were seeded into each ring. A tissue culture plastic (TCP) control was always included. In the case of experiments performed with keratinocytes and HCEC cells the TCP was coated with the appropriate proteins (see section 2.2.7 and 2.2.10). Once seeded, cells were placed in the incubator for 1 hour and then extra medium was gently added. Cells were cultured in direct contact with PNVP for 4 days.

2.5.2 Culture of cells in indirect contact with PNVP polymers.

A cartoon illustrating the experimental set up is shown in figure 2.1.B. Basically cells were seeded at 5 x 10^4 cells per well in 10 μl of medium into standard 24 well Costar™ culture plates. PNVP hydrogels were placed into ThinCert™ PET tissue culture inserts with 8 μm pores either suspended above the cells in the culture well. A TCP control was always included. In the case of experiments performed with keratinocytes and HCEC cells the TCP was coated with the appropriate proteins (see section 2.2.7 and 2.2.10). Once seeded, cells were placed in the incubator for 1 hour and then extra medium was gently added. Cells were cultured in the presence of PNVP for 4 days.

2.5.3 Effect of hydrogel-conditioned media on fibroblast viability.

PNVP hydrogels were placed into wells in a 24 well plate. 1 ml of appropriate media was added (serum free DMEM or fibroblast culture medium). The plates were placed into an incubator at 37°C, 5% CO_2 for 4 days. The now conditioned media was transferred to cells plated out at a density of 5 x 10^4 cells in fibroblast culture medium 24 hours previously. Cells were cultured in PNVP conditioned medium for 4 days.

2.6 Cell viability, proliferation and differentiation assays.

Materials.

PBS, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Propan-2-ol, concentrated hydrochloric acid (HCl), 2-ethoxyethanol (Cellosolve). 4',6-Diamidino-2-phenylindole (DAPI), Urea, SDS.

Acidified Isopropanol 25 µl concentrated HCl was added to 20 ml isopropanol. This solution was stored until needed.

DAPI Solution Stock solution consisting of 1 mg.ml^{-1} of DAPI in PBS was prepared. 10 µl aliquots were frozen at -20°C until needed. These aliquots were then diluted 1:1000 in PBS immediately prior to experimental use.

Lysis Buffer 48 g of urea and 0.04 g of SDS were dissolved in 100 ml of PBS.

MTT solution MTT was dissolved in PBS to a final concentration of 0.5 mg.ml^{-1}. The solution was made fresh for each experiment.

The plate readers used in these experiments were Dynex Technologies MRXII microplate reader attached to a PC running Revelation 2.0 software. Fluorescent plate reader Bioteck Flx800 microplate fluorescence reader attached to a PC running KC4 v3.3 software. The epifluorescence microscope was obtained from Leica Microsystems (UK) Ltd, UK. Photographs were taken with a Hamamatsu Orca camera from Hamamatsu Photonics UK Ltd, UK. Openlab 3.1.7 software and Openlab filter- and shutter-controlling hardware were obtained from Improvision, UK.

2.6.1 DAPI DNA assay.

Urea, DAPI and SDS were obtained from Sigma, UK.

Cells were washed with PBS, 1 ml of lysis buffer was added to the cells and incubated for 1 hour at 37°C. Samples were collected into micro-centrifuge tubes. 100 µl of sample was added to 100 µl of DAPI solution and fluorescence was read using a fluorescent plate reader (λex = 360/340 nm λem = 460/440 nm).

2.6.2 Involucrin assay.

Goat serum, IgG1 anti-human involucrin, mouse isotype involucrin IgG goat anti-mouse IgG-FITC conjugate and sodium hydroxide (NaOH) were obtained from Sigma, UK. 10% phosphate-buffered formaldehyde was obtained from Genta Medical, UK. Methanol was obtained from Fisher, UK.

At the end of the culture period cells were fixed in 10% buffered formaldehyde for 10 minutes at room temperature. Cells were then treated on ice with absolute methanol for 5 minutes to permeabilise the cell membrane. The cells were then washed three times in PBS and incubated in 10% (v/v) solution of goat serum in PBS. The goat serum was then removed and the cells were washed in PBS. The wells were then incubated for 45 minutes with 18 μg.ml^{-1} mouse primary antibody IgG1 antihuman involucrin in PBS. In order to control for the binding specificity of the primary antibody, control wells were incubated with 18 μg.ml^{-1} of a mouse IgG1 Kappa antibody in PBS. After washing with PBS, all of the wells were incubated with a secondary antibody, goat anti-mouse IgG – FITC conjugate (Fab specific) in PBS (1:500) for 45 minutes. To obtain quantitative values, the fluorescent label was eluted by the addition of 0.1 M NaOH at 37°C for 1 hour. The fluorescence of 100 μl of each sample was read in a Bioteck Flx800 microplate fluorescence reader attached to a PC running KC4 v3.3 software, using an excitation wavelength 360/340 nm and emission wavelength 460/440 nm.

Microscopy was performed within 24 hours of staining and DAPI-stained plates were kept in the dark at 4°C prior to microscopy. Fluorescent emission was visualised using a Leica epifluorescence microscope λex = 495 nm, λem = 515 nm (for FITC/involucrin visualisation) and λex = 358 nm, λem = 461 nm (for DAPI/nuclei visualisation) and images were captured using a Hamamatsu Orca camera attached to an Apple computer (MAC OS 9.2) running Openlab 3.1.7 software.

2.6.3 MTT-eluted stain assay.

MTT and Cellosolve were obtained from Sigma, UK. Concentrated HCl was obtained from VWR. Propan-2-ol was obtained from Fisher, UK. PBS tablets were obtained from Oxoid, UK.

Samples were washed gently with PBS. For 24 well plates 1 ml of MTT solution was used per well, 12 well plates used 1.5 ml of MTT solution per well. The plates were incubated with MTT solution for 40 minutes at $37^{\circ}C$, 5% CO_2 in a humidified atmosphere. The MTT solution was subsequently removed and for fibroblast cultures acidified isopropanol was used to elute the formazan product from the cells. For keratinocyte, HaCaT and HCEC cultures and experiments where cells were grown in direct contact with the PNVP hydrogels, 2-ethoxyethanol was used to elute the formazan from the cells. 75 μl of the sample was placed in a 96 well plate and the optical density was read in a Dynex Technologies MRXII microplate reader attached to a PC running Revelation 2.0 software at 540 nm and referenced at 630 nm.

2.7 Collagen gels.

Materials.

NaOH was obtained from sigma, UK. Collagen from Rat tail was obtained from Fluka, UK. Glacial acetic acid was obtained from VWR, UK. DMEM and FCS were obtained from Biowest Biosera, UK.

Acetic Acid solution 0.1 M Glacial acetic acid (17.4 M) was diluted in distilled water to produce a 0.1 M solution. This was then taken into a class II cell culture hood and filter sterilised kept in a sterile bottle until needed.

Collagen I solution (5 mg.ml^{-1}) Rat tail type I collagen was placed into a sterile bottle/container containing a sterile stirring bar and made up to 5 mg.ml^{-1} by adding sterile 0.1 M acetic acid. This was kept cool and stirred slowly until all the collagen had dissolved, and the stock solution was kept at 4°C until needed (no more than 3 weeks).

Sodium Hydroxide solution (1 M) Sodium hydroxide pellets were dissolved in distilled water to produce a 1 M solution. This was then taken into a class II cell culture hood and filter sterilised and then kept in a sterile bottle until needed.

Collagen gels were prepared with a final collagen concentration of 2.7 mg.ml^{-1}. 3.6 ml of 5 mg.ml^{-1} collagen solution was placed into a chilled universal. 2.1 ml DMEM was added and gently mixed. 1 M NaOH was added dropwise to neutralise the acetic acid. 0.9 ml FCS (containing if needed the appropriate number of cells) was then added to the collagen solution and mixed well. 350 µl of collagen solution was used per well of a 24 well plate. The plate was gently rocked to ensure that the gels spread out uniformly. The gels were placed into an incubator to set. After 24 hours the gels were released from the side of the wells using a fine pipette tip (p20) and 1 ml of medium was added to the wells.

2.7.1 Fibroblast monoculture collagen gels .

5 x 10^4 cells per gel were suspended in the FCS used to make the collagen gel. After the gel set 1 ml of fibroblast culture medium was gently added to the wells.

2.7.2 Keratinocyte monoculture collagen gels.

Collagen gels were prepared as per section 2.7 without cells in the FCS. After the gels had set 5×10^4 keratinocytes (P1 or 2) were placed on the top of the gel in 50 μl of Greens medium and allowed to attach for 20 minutes at 37°C. 1 ml of Greens medium was then gently added to the wells.

2.7.3 Fibroblast-keratinocyte co-culture collagen gels.

Collagen gels were prepared as per section 2.7 with 1.25×10^4 fibroblasts per gel in the FCS. After the gels had set 3.75×10^4 keratinocytes (P1 or 2) were placed on the top of the gel in 50 μl of Greens medium and allowed to attach for 20 minutes at 37°C. 1 ml of Greens medium was then gently added to the wells.

2.7.4 Effect of βAPN and 4-MU on the contraction of co-cultured collagen gels.

Co-cultured collagen gels were prepared as per section 2.7.3. Before the gels were released from the sides of the wells all the media was removed from the gels. Once the gels had been released 1 ml of media supplemented with βAPN $50 - 800$ μg.ml^{-1} or 4-MU $0.125 - 4$ mM was added to the wells. Every 24 hours from this point on all the media was removed from each well and the collagen gels. The gels were photographed for image analysis (see section 2.9) and 1 ml of fresh βAPN or 4MU supplemented media was added. The experiments were run over 6 days.

2.7.5 Effect of 4-MU on the contraction of mono and co-cultured collagen gels.

Collagen gels were prepared seeded with mono and co-cultures of fibroblasts and keratinocytes as per sections 2.7.1-3. Before the gels were released from the sides of the wells all the media was removed from the gels. Once the gels had been released 1 ml of media supplemented with 2 mM 4-MU was added to the wells. Every 24 hours from this point on all the media was removed from each well and the collagen gels. The gels were photographed for image analysis (see section 2.9) and 1 ml of fresh βAPN or 4MU supplemented media was added. The experiments were run over 6 days.

2.7.6 Effect of βAPN and 4-MU loaded hydrogels on collagen gel contraction.

4-methyl umbelliferone sodium salt was obtained from Sigma, UK. P(NVP-co-DEGBAC) and P(NVP-co-DEGBAC-co-AA) xerogels were produced as per section 2.1.7. PBS was produced as per section 2.2.

4-methyl umbelliferone solution 4-methyl umbelliferone sodium salt was dissolved in PBS (produced as per section 2.2) to produce solutions of 1 mM and 4 mM these solutions were then filter sterilised.

β-aminopropionitrile fumarate solution 3-aminopropionitrile fumarate was dissolved in PBS (produced as per section 2.2) to produce solutions of 1000 $\mu g.ml^{-1}$ and 200 $\mu g.ml^{-1}$ these solutions were then filter sterilised.

Sterile xerogels were placed into 30 ml universals containing 25 ml of βAPN or 4-MU solution at the appropriate concentration. These were placed onto a slowly revolving blood rolling table at room temperature for 2 days, until the gels were hydrated. The gels were then suspended in ThinCert™ cell culture inserts above collagen gels that had just been released from the sides of the well. 1 ml of Greens medium was gently added to each well. After 24 hours the medium was completely removed from the wells and the cell culture inserts. The inserts were placed into a fresh sterile 24 well plate while the collagen gels were photographed for image analysis. The inserts were then re-suspended above the gels and 1 ml of fresh Greens medium added. This procedure was repeated every 24 hours for 6 days.

2.7.7 Effect of 4-MU loaded hydrogels, changed regularly, on collagen gel contraction.

Hydrogels loaded with 4mM 4-MU were produced as per section 2.7.6.

The hydrogels were suspended in ThinCert™ cell culture inserts above collagen gels that had just been released from the sides of the well. 1 ml of Greens medium was gently added to each well. After 24 hours the medium was completely removed from the wells and the cell culture inserts. The inserts were placed into a fresh sterile 24 well plate while the collagen gels were photographed for image analysis. The inserts

were then re-suspended above the gels and 1ml of fresh Greens medium added. This procedure was repeated every 24 hours for 6 days with the 4-MU hydrogels being changed for fresh 4-MU loaded hydrogels every second day.

2.8 Preparation of tissue-engineered composites.

The protocol used was developed within our laboratory and reported by Ghosh (Ghosh, Boyce et al. 1997). It was subsequently modified by Chakrabarty (Chakrabarty, Dawson et al. 1999).

2.8.1 Sterilisation of donor skin.

The skin was received from theatre and stored in PBS supplemented with penicillin/streptomycin (penicillin 50 I.U.ml^{-1} and streptomycin 50 μg.ml^{-1}) and Fungizone (313 μg.l^{-1}) at 4°C for up to 14 days. The skin was then immersed in a sterile mixture of 50% glycerol: 50% PBS (v/v) for 4 hours followed by 85% glycerol: 15% PBS for 18 hours. The skin was finally placed into 100% glycerol for 26 hours. The skin was then sent for further ethylene oxide sterilisation.

The skin was removed from 100% glycerol (section 2.6.1) and allowed to drip dry before any excess glycerol was removed by gently dabbing the skin with absorbent paper towel. The skin was the placed into autoclave bags, labelled and sealed with autoclave tape. Ethylene oxide sterilisation was performed at the Central Sterile Services Department (CSSD) of Leicester Royal Infirmary by the Sterivit^{TM} procedure. The bagged skin samples were placed onto racks and exposed to 15% ethylene oxide, 85% carbon dioxide (a concentration of 200 mg ethylene oxide per litre) at 55°C and a pressure of 5.5 atmospheres for 30 minutes. After sterilisation, the samples were stored at room temperature for at least 3 days to allow aeration and dissipation of any remaining ethylene oxide gas. Ethylene oxide treated skin was then stored, in sealed autoclave bags, at room temperature until needed.

2.8.2 De-epidermisation of donor skin.

All procedures were performed in a class II culture hood. The sterilised skin was removed from the sealed autoclave bags and placed into 100 ml sterile plastic containers containing sterile PBS which was then incubated at 37°C for at least 2 days. The skin was then removed from the PBS solution and placed into 100 ml sterile plastic containers containing sterile 1 M sodium chloride solution. The skin

was then incubated overnight at 37°C (typically 14 – 18 hours). At this stage there was visible separation of the dermis from the epidermis. Epidermis that was still attached to the dermis was gently detached by gentle scraping with a blunt-ended spatula. The de-epidermised dermis (DED) was then washed twice with PBS and incubated in 10% Greens medium at 37°C for a minimum of 48 hours. This had a threefold purpose i) ensured that any sodium chloride solution remaining in the DED was washed out ii) allowed the DED to become saturated with culture medium and iii) provided a rough sterility check for the DED (in the presence of infection, the medium becomes more acidic and therefore the phenol red pH indicator in the medium changes colour from crimson red to yellow). For any one experiment, DED from a single patient was used and wherever possible cut from the same sheet. This was to try to reduce inter-patient variation in skin characteristics and differences in thickness. For experimental use the DED was cut into squares with dimensions of approximately 1.5 x 1.5 cm and the reticular surface was placed on the bottom of the culture well.

2.8.3 Production of tissue-engineered skin composites.

Rings and grids were manufactured from medical grade stainless steel by the Department of Medical Physics, Royal Hallamshire Hospital, Sheffield. 6-well tissue culture plates were Costar™ obtained from Corning Inc., USA.

Following skin rehydration and de-epidermisation, the DED was cut with a scalpel to squares of approximately 1.5 x 1.5 cm and placed into the wells of a six well plate with the papillary dermis facing upwards. A chamfered metal ring (internal diameter 1 cm) was placed in the centre of each piece of DED and was gently pressed down to ensure a watertight seal and thus prevent leakage of the cell suspension. 10% Greens media was added to the culture well outside the ring to allow the seal to be tested. Keratinocytes and fibroblasts were seeded into the centre of the ring (keratinocytes 3×10^5 in 300 µl 10% Greens, fibroblasts 1×10^5 in 200 µl 10% Greens). The culture plates were then incubated at 37°C for 48 hours with the media inside the ring being changed twice during this period prior to raising to air-liquid interface (ALI). After 48 hours, the media and the seeding rings were removed. A no.22 scalpel blade was used to cut around the seeded area and the unseeded DED was discarded. A sterile

stainless steel grid was introduced to each well and the composite was placed on this grid seeded surface uppermost. Fresh 10% Greens medium, or Greens medium plus 4-MU was placed into each well until it just touched the reticular surface each composite whilst leaving the seeded papillary surface exposed to the air. The composites were cultured at 37°C in a humidified 5% CO_2 atmosphere for 28 days. The cell culture medium was changed every 3 - 4 days. A cartoon illustrating the experimental set up for a seeded composite at an ALI is shown in figure 2.2 below.

Figure 2.2 Diagram showing schematic for the culture of tissue engineered skin composite at an air-liquid interface.

2.8.4 Effect of 4-MU on contraction of tissue engineered skin composite.

4-Methyl umbelliferone (4-MU) solution 4-MU sodium salt was dissolved in DMEM to produce a stock concentration of 0.1 M. This solution was filter sterilised and made fresh for each experiment.

To examine the effect of 4-MU on the contraction of the skin composite the composites were prepared as per section 2.8.3 and the medium was supplemented with 4-MU to produce final concentrations of 0.5, 1 and 2 mM. Every 7 days the composites were photographed for image analysis (see section 2.9).

2.9 Image analysis.

The collagen gels and composites were photographed using a Nikon 990 Coolpix digital camera. In each case the plate was placed alongside a scale bar so that the captured digital image could be calibrated and thus the area of the composite be calculated. The camera was placed directly above the plate (without its lid) inside the class II culture hood to maintain sterility but to minimise distortion of the image. The images were imported into ImageJ software and the scale bar used to calibrate the image. The zoom facility in the programme was used to increase the size of each image to reduce observer error. A computer mouse was used to trace the edge of the composite freehand. The software then automatically calculated the area of this plot, relative to the calibration derived from the scale bar. The area of the composite on day 0 (when it was raised to an air-liquid interface) was designated 100% and all changes in area were expressed relative to this initial measurement.

2.10 Histology.

Materials.

10% phosphate-buffered formaldehyde was obtained from Genta Medical, UK.

Carazzi's Haematoxylin: 5 g Haematoxylin (CI 75290) was dissolved in 800 ml distilled water, 50 g aluminium potassium phosphate, 0.6 g potassium iodate and 200 ml glycerol was then added to the solution. The solution was mixed well and then filtered.

Eosin: A 1% w/v solution of eosin (CI 45280) was adjusted to pH 6.3 with dilute HCl.

Acid/alcohol: 1% v/v HCl in 70% ethanol.

Composites were placed into 10% phosphate-buffered formaldehyde at room temperature, which were subsequently embedded in paraffin wax. 5 µm sections were cut, mounted and stained using haematoxylin and eosin by Dr Christopher Layton, Department of Histopathology, Northern General Hospital, Sheffield.

2.10.1 Carazzi's Haematoxylin and Eosin staining.

This stain was used to show the cellular architecture/interactions within the tissue. Haematoxylin stains acidic structures such as cell nuclei purple/blue. Eosin stains proteins and other basic structures pink.

The wax covering the sections was removed by submerging the slides in xylene for approximately 10 minutes. The xylene was then removed by taking the slides through descending grades of alcohol i.e. 90%, 75% and 50% and then into water. The slides were stained with haematoxylin for 3 minutes and then excess stain was removed by rinsing the slides in tap water. The haematoxylin stains all cellular material not just the nuclei. In order to remove the haematoxylin from the cytoplasm of the cell, the slides were differentiated in 1% acid/alcohol for approximately 20 seconds. Differentiation was confirmed microscopically then sections were washed in running tap water. The slides were then stained with eosin for approximately 20 seconds. Dehydration was achieved by taking the slides through ascending grades of alcohol i.e. (50%, 75% and 90%). The slides were then placed back into xylene and cover slips were applied.

2.10.2 Histological scoring of skin composites.

In order to assess the effect of 4-MU on the appearance of the reconstructed skin composites, a scoring system was used. This was the scoring system used by Miss Caroline Harrison and is an adaptation of a previous scoring system devised by Ms. R. Dawson, research technician, University of Sheffield; Dr C. Layton, Department of Histopathology, Northern General Hospital, Sheffield and Professor S. MacNeil. Scoring was carried out by 5 independent observers within the MacNeil group, who each have significant experience examining composite histology. Photomicrographs of the H&E stained composites were given a random anonymous reference number. Photomicrographs were taken using a Motic digital microscope (B5 professional series) attached to a PC running Motic Images Advanced 3.1 software. The digital photomicrographs were examined on PC monitors by the observers, thus allowing the zoom feature to be used during the scoring procedure.

Skin scoring system

AREA	DESCRIPTION	SCORE
Keratin		
	Normal keratin	A
	Vacuolation within the keratinised layers	B
	Thin layer of poorly adherent/fragmented keratin	C
	Non-existent	D
Keratinocyte layer		
	Good, organised, thick with differentiation	A
	Either parakeratosis or disordered keratinocyte differentiation	B
	Thin (2-3 cells), organised, continual	C
	Monolayer, patchy	D
	Non-existent	E
Dermo-Epidermal Junction		
	Fully attached, with rete ridges present	A
	Attached but no rete ridges	B
	Partial attachment	C
	No attachment	D
Dermis		
	Increased dermal density	A
	Normal, organised pattern of reticular and papillary dermis	B
	Disorganised, small gaps / holes	C
	Bland collagen tissue, large gaps / holes	D
	Disintegrating	E
Fibroblasts		
	Large number, enhanced proliferation	A
	Average number	B
	Few, reduced proliferation / poor survival	C
	Non-existent	D

2.11 SDS-PAGE electrophoresis and protein staining with Coomassie Brilliant Blue™.

Materials.

Tris-HCl, Tris, β-mercaptoethanol, glycerol, bromophenol blue, 30% w/v acrylamide/0.8% w/v N,N'-methylene-bis-acrylamide, N,N,N',N'-Tetremethylethylenediamine (TEMED), ammonium persulphate (APS), glycine, glacial acetic acid were obtained from VWR, UK. Coomassie Brilliant Blue R250, EDTA, SDS, and SDS-7B molecular weight ladder were obtained from Sigma, UK. The proteins used in the SDS-7B molecular weight ladder were α_2 macroglobulin (185 kDa), β-glactosidase (115 kDa), fructose 6-phosphate kinase (84 kDa), pyruvate kinase (61.5 kDa), fumarase (55 kDa), lactic dehydrogenase (36 kDa) and triosephosphate isomerise (31 kDa). DMEM, DMEM + 10% FCS, FCS Methanol was obtained from Fisher, UK.

Coomassie Brilliant Blue 0.25% (w/v) Coomassie Brilliant Blue™, 40% (v/v) methanol, 7% (v/v) acetic acid, deionised water.

Coomassie Brilliant Blue Destain 40% (v/v) methanol, 7% (v/v) acetic acid, deionised water.

Loading buffer 0.5 ml β-mercaptoethanol, 2 ml glycerol, 1.5 g SDS, 0.01 g bromophenol blue, 0.6 ml 1.25 M Tris HCl pH 6.8, 6.9 ml ddH$_2$O.

Overlay 12.5 ml 1.875 M tris pH 8.8, 36.5 ml deionised water and 1 ml 10% w/v SDS.

Resolving gel (7%) 2.3 ml of 30% w/v acrylamide/0.8% w/v N,N'-methylene-bis-acrylamide, 2.5 ml of 1.875 M Tris buffer pH8.8, 4.92 ml deionised water, 0.2 ml 10% w/v SDS, 10 μl TEMED and 70 μl 10%w/v APS.

Running buffer 3 g Tris, 14.4 g Glycine, 2 g SDS, 0.75 g EDTA dissolved in 800 ml of ddH$_2$O and make up the volume to 1000 ml with ddH$_2$O. This was stored at 4°C until required.

Stacking gel 0.76 ml 30% w/v acrylamide/0.8% w/v N,N'-methylene-bis-acrylamide, 0.5 ml 1.25 M tris pH 6.8, 3.61 ml deionised water, 0.1 ml 10% w/v SDS, 5 μl TEMED, 25 μl 10% w/v APS.

All samples (DMEM, DMEM + 10% FCS, FCS, and P(NVP-co-DEGBAC) soaked in DMEM, DMEM + 10% FCS and FCS) were placed into micro-centrifuge tubes with 1:1 v/v loading buffer for 5 minutes at 100°C. The SDS-7B molecular weight ladder was also placed into a micro-centrifuge tube with 1:1 v/v loading buffer for 5 minutes at 100°C.

The resolving gel solution was placed into a multicasting chamber leaving a 3 cm gap at the top. Overlay solution was then gently added to fill the chamber and the gel was allowed to polymerise for approximately 1 hour. The overlay was then gently poured off and stacking gel solution added. A lane comb was inserted into the stacking gel solution to form the gel lanes. The stacking gel was allowed to polymerise for approximately 1 hour. Once the stacking gel had polymerised the well comb was carefully removed and the denatured samples added, including ladder. The electrophoresis unit was placed into the electrophoresis tank and submerged in running buffer. The proteins were separated at a constant voltage of 200 V for 1 hour. The stacking gel was then removed and the resolving gel placed into 1 M acetic acid. The acetic acid was removed and the gel placed into Coomassie Brilliant Blue™ solution for 1 hour. This solution was removed and destain added. The gel was left in destain for 2 hours and then changed. The destain was then left overnight (approximately 12 - 18 hours) and changed again. The gel was then placed onto a flat bed scanner attached to a PC running Epson scansmart software.

2.12 β-aminopropionitrile (βAPN) release.

Materials

2,4,6-trinitrobenzenesulfonate 5% (w/v) in H₂O (TNBS), sodium bicarbonate, 3-aminopropionitrile fumarate (βAPN) and bovine serum albumen (BSA) were obtained from Sigma, UK. Concentrated hydrochloric acid was obtained from VWR. P(NVP-co-DEGBAC) and P(NVP-co-DEGBAC-co-AA) xerogels were produced as per section 2.1.7. PBS was produced as per section 2.2. Plate reader used for βAPN release assay was a Bioteck ELx800uv microplate reader attached to a PC running KCJunior software optical density was measured at 320 nm and referenced at 630 nm.

β-aminopropionitrile fumarate solution 3-aminopropionitrile fumarate was dissolved in PBS (produced as per section 2.2) to produce solutions of 1000 μg.ml⁻¹ and 200 μg.ml⁻¹.

Sodium bicarbonate solution 0.1 M solution was produced by dissolving sodium bicarbonate pellets in water the pH was then adjusted to pH8.5 by the addition of concentrated hydrochloric acid.

TNBS solution 0.01% solution of TNBS was produced by diluting 2 μl of TNBS per ml of sodium bicarbonate solution.

2.12.1 Loading of PNVP xerogels with βAPN solution.

Xerogels were placed into 30 ml universals containing 25 ml of βAPN solution at the appropriate concentration (200 or 1000 μg.ml⁻¹). These were placed onto a slowly revolving blood rolling table at room temperature for 2 days, until the gels were hydrated.

2.12.2 Release of βAPN from PNVP hydrogels.

βAPN loaded hydrogels were produced as per section 2.12.1. The hydrogels were gently removed from the universal and gently dabbed with absorbent paper to remove any excess solution. The hydrogels were then placed into individual wells of

a 48 well plate and 1 ml of PBS gently added to each well. After 24 hours the PBS was completely removed and stored for later analysis. 1 ml of fresh PBS was then added to each well and this was repeated every 24 hours for 6 days.

2.12.3 Detection of βAPN in PBS.

10 μl of the PBS stored from 2.12.2 was transferred to a 96 well plate 90 μl sodium carbonate buffer was added. 50 μl of TNBS solution was added to each well and the plate left in the dark at room temperature for 2 hours. The absorbance was read at 320 nm in a Bioteck ELx800uv microplate reader attached to a PC running KCJunior software. The absorbance was then converted to βAPN concentration using the calibration graph shown in figure 2.3 below.
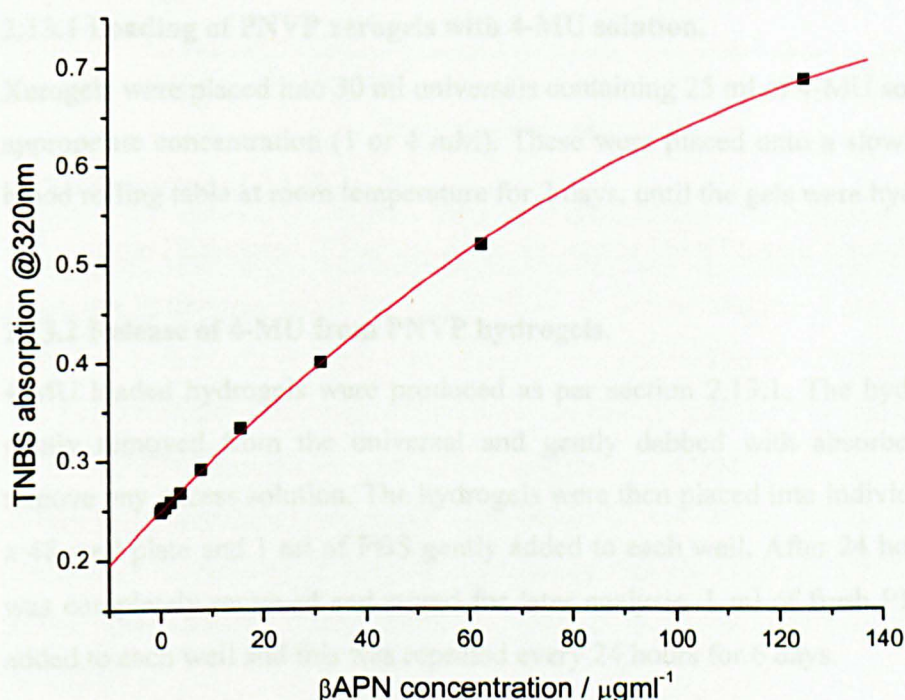
Figure 2.3 Final βAPN calibration curve, samples diluted down to final concentrations using $Na(CO_3)_2$ buffer pH 8.5 (n=6). All data expressed as mean ± SEM.

2.13 4-methyl umbelliferone (4-MU) release.

Materials.

4-methyl umbelliferone sodium salt was obtained from Sigma, UK. P(NVP-co-DEGBAC) and P(NVP-co-DEGBAC-co-AA) xerogels were produced as per section 2.1.7. PBS was produced as per section 2.2. The plate reader used for 4-MU release assay was a Bioteck Flx800 microplate fluorescence reader attached to a PC running KC4 v3.3 software. (λex = 360/340 nm λem = 460/440 nm).

4-methyl umbelliferone solution 4-methyl umbelliferone sodium salt was dissolved in PBS (produced as per section 2.2) to produce solutions of 1 mM and 4 mM.

2.13.1 Loading of PNVP xerogels with 4-MU solution.

Xerogels were placed into 30 ml universals containing 25 ml of 4-MU solution at the appropriate concentration (1 or 4 mM). These were placed onto a slowly revolving blood rolling table at room temperature for 2 days, until the gels were hydrated.

2.13.2 Release of 4-MU from PNVP hydrogels.

4-MU loaded hydrogels were produced as per section 2.13.1. The hydrogels were gently removed from the universal and gently dabbed with absorbent paper to remove any excess solution. The hydrogels were then placed into individual wells of a 48 well plate and 1 ml of PBS gently added to each well. After 24 hours the PBS was completely removed and stored for later analysis. 1 ml of fresh PBS was then added to each well and this was repeated every 24 hours for 6 days.

2.13.3 Detection of 4-MU in PBS.

25 μl of the PBS stored from 2.13.2 was transferred to a 96 well plate. To this was added 175 μl of PBS, the fluorescence was then read using an excitation wavelength of 360/340 nm and emission wavelength of 460/440 nm in a Bioteck Flx800 microplate fluorescence reader attached to a PC running KC4 v3.3 software. The

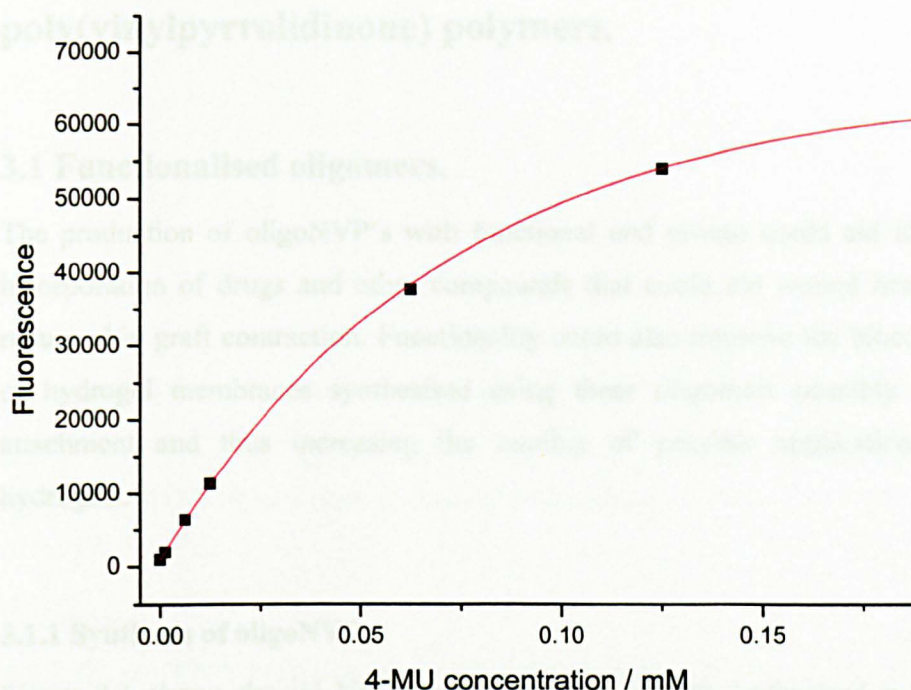fluorescence was then converted to 4-MU concentration using the calibration graph shown in figure 2.4.

Figure 2.4 Calibration curve used for drug release experiments. Excitation wavelength 360/340 nm, emission monitored 460/440 nm. Data expressed as mean ± SEM of n=6 samples.

2.14 Statistics.

Statistics used during this project were Students' T-Test and 1-way ANOVA with Bonferroni Correction. Statistical significance was assessed by $p < 0.05$. GraphPad InStat 3.06 software from GraphPad software Inc, USA was used for statistical analysis.

3. Synthesis and characterisation of poly(vinylpyrrolidinone) polymers.

3.1 Functionalised oligomers.

The production of oligoNVP's with functional end groups could aid the chemical incorporation of drugs and other compounds that could aid wound healing and/or reduce skin graft contraction. Functionality could also improve the biocompatibility of hydrogel membranes synthesised using these oligomers possibly aiding cell attachment and thus increasing the number of possible applications of these hydrogels.

3.1.1 Synthesis of oligoNVP.

Figure 3.1 shows the ^{1}H NMR spectrum of oligoNVP synthesised in bulk. GPC measurements of the oligoNVP gave a weight average molecular weight (Mw) of 251 g.mol^{-1} with a polydispersity of 1.02 indicating that the oligomers formed primarily as dimers with a small proportion of trimers being produced. Reducing the amount of initiator in the reaction system and possibly adding a small amount of solvent to retard the reaction slightly could increase the chain length. The wide peaks are indicative of the presence of polymer instead of monomer.
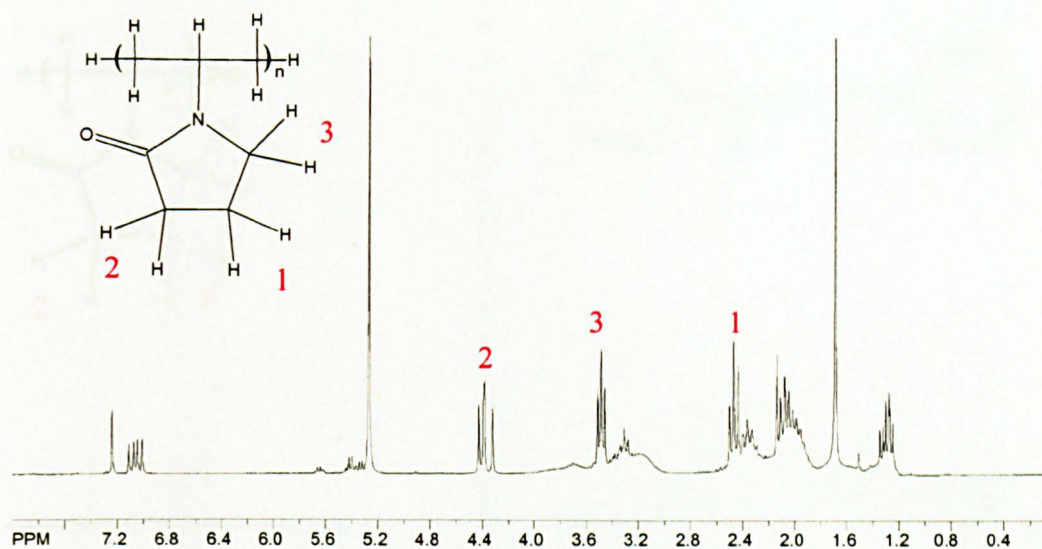
Figure 3.1 ^{1}H NMR spectrum of oligoNVP in CHCl$_3$.

3.1.2 OligoNVP functionalised with benzene dithiobenzoate.

An attempt to produce oligoNVP using RAFT polymerisation using BDTB as the RAFT agent gave the NMR spectrum shown in figure 3.2. The yield was 1.63% unfortunately this meant that after structural verification there was insufficient sample for further GPC analysis. This low yield meant that this synthesis route would not be practical for the large quantities of oligomers needed for subsequent membrane synthesis. Again the wide peaks seen in this spectrum are indicative of the presence of polymeric species.
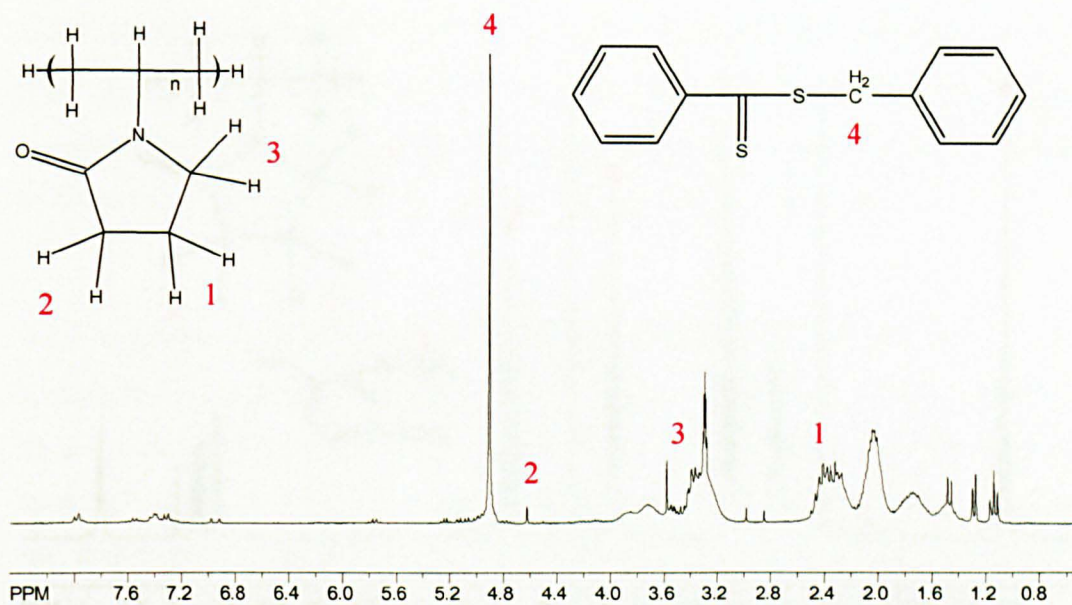
Figure 3.2 ^{1}H NMR in MeOH of oligoNVP polymerised in the presence of the RAFT agent BDTB.

3.1.3 OligoNVP functionalised with silyl enol ether.

The polymerisation of NVP in the presence of the silyl enol ether MPTMSE gave the NMR spectrum shown in figure 3.3. The GPC analysis of this functionalised oligoNVP gave $\underline{Mw}$ of 266 g.mol^{-1} with a polydispersity of 1.04. This is indicative of the presence of NVP dimers and trimers again larger oligomeric fragments would be required for membrane synthesis.
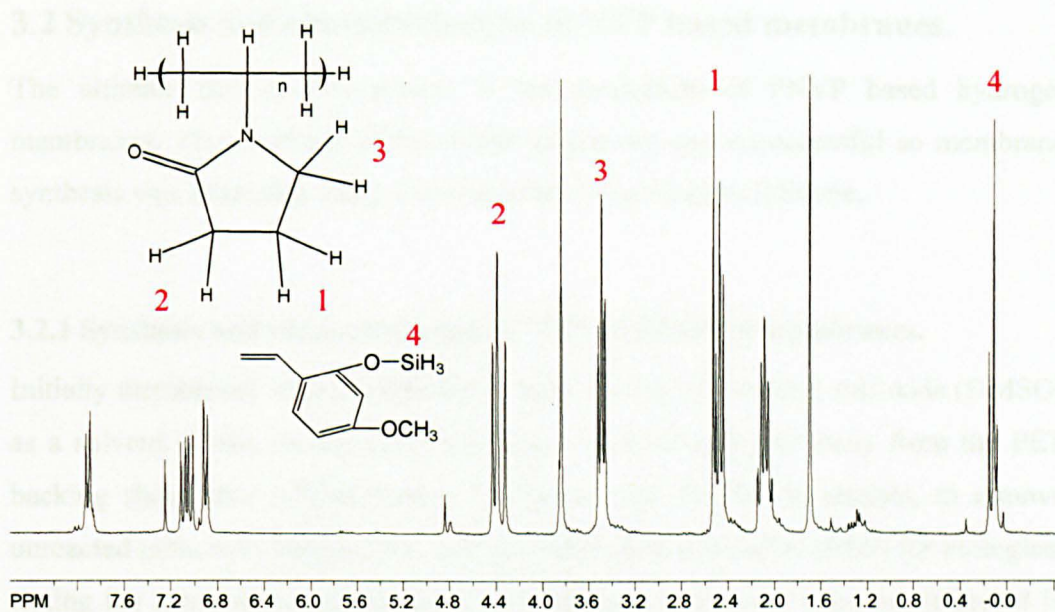
Figure 3.3 ^{1}H NMR spectrum in CDCl$_3$ of NVP polymerised with MPTMSE.

3.1.4 Summary of results.

Living polymerisation routes were used in an attempt to maintain a narrow polydispersity. The synthesis of oligomers via RAFT polymerisation with benzyl dithiobenzoate was unsuccessful, both in the presence and absence of the solvent dioxane due to very low conversions. The synthesis of the NVP oligomers functionalised with the silyl enol ethers was also unsuccessful, again due to the unstable radical resulting in very low conversions, both in the presence and absence of the solvent toluene. The NVP radical is not very stable and it is therefore likely that in the presence of solvent the radical transferred to the solvent molecules.

3.2 Synthesis and characterisation of NVP based membranes.

The ultimate aim of this project is the production of PNVP based hydrogel membranes. The synthesis of functional oligomers was unsuccessful so membrane synthesis was attempted using the monomer 1-vinyl-2-pyrrolidinone.

3.2.1 Synthesis and characterisation of NVP-co-EGDMA membranes.

Initially membranes were synthesised in the presence of dimethyl sulfoxide (DMSO) as a solvent. These membranes were easy to handle and peel away from the PET backing sheet after polymerisation. However once swollen in ethanol, to remove unreacted monomer, initiator etc., and phosphate buffered saline (PBS) for biological testing the membranes became extremely difficult to handle. This was reflected in the equilibrium water content (EWC). These hydrogels had a EWC of 98.25% ± 0.155% as shown in table 3.1 and illustrated in figure 3.4 below resulting in problems handling these membranes.

Table 3.1 EWC measurements. Measurements performed on polymers swollen in PBS. Dried at 50°C in a vacuum oven for 24 hours (n=6). Measurements expressed as mean ± standard error of mean (SEM).

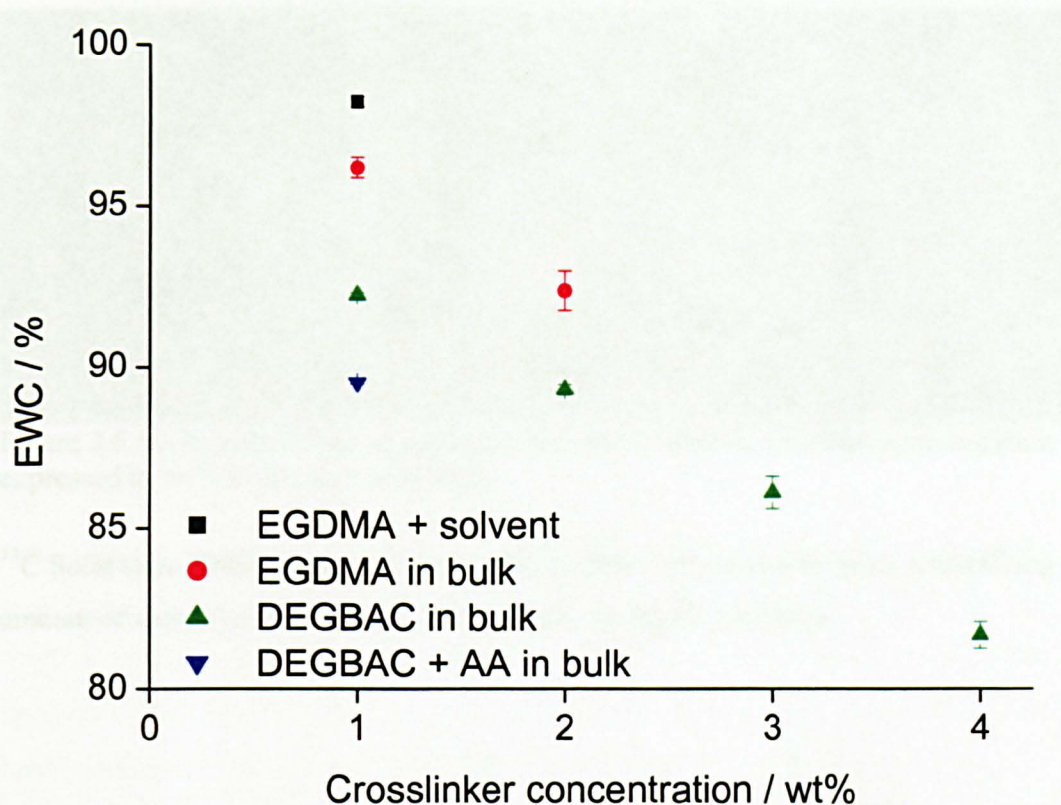| Polymer	EWC in PBS @ room temperature / %	SEM / %
NVP 1 wt% EGDMA +DMSO	98.3	0.2
NVP 1 wt% EGDMA	96.2	0.3
NVP 2 wt% EGDMA	92.3	0.6
NVP 1 wt% DEGBAC	92.2	0.1
NVP 2 wt% DEGBAC	89.3	0.2
NVP 3 wt% DEGBAC	86.1	0.5
NVP 4 wt% DEGBAC	81.7	0.4
NVP 1 wt% DEGBAC 1 wt% AA	89.5	0.1

Figure 3.4 Effect of synthesis conditions, presence of solvent, crosslinker, crosslinker concentration and addition of acrylic acid on equilibrium water content of membranes swollen in phosphate buffered saline measurements expressed as mean ± SEM (n=6).

To reduce the EWC the next batch of membranes were synthesised in bulk, i.e. without solvent. This did reduce the EWC to 96.18% and raise the membrane material properties. However they still did not have the handle-ability required of a wound dressing. Increasing the concentration of EGDMA (1-5 wt%) in the membranes produced opaque, phase separated membranes as illustrated in figure 3.5. Once hydrated these membranes were significantly stiffer than the original 1 wt% EGDMA membranes. However, they had little material integrity and flaked easily when removed from liquid. Therefore the only membrane where EWC measurements were possible was the NVP 2 wt% EGDMA membrane.
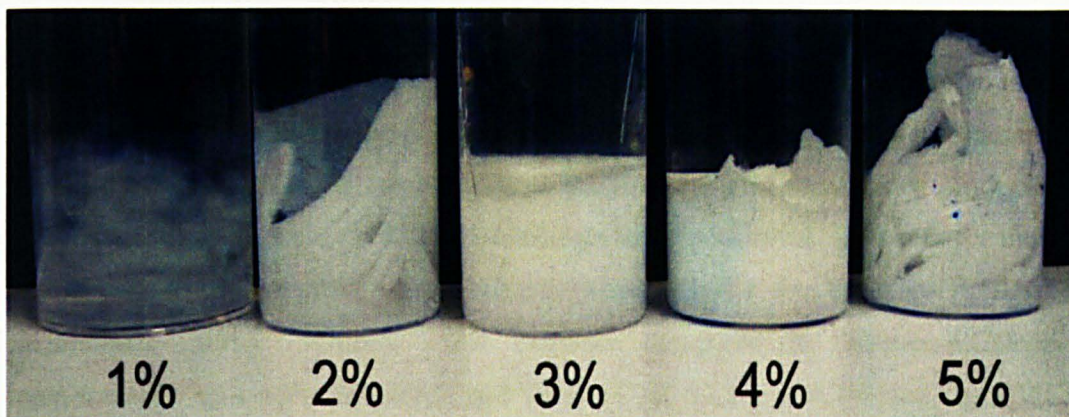
Figure 3.5 NVP-co-EGDMA membranes swollen in ethanol. EGDMA concentration expressed as wt% in the monomer feed.

^{13}C Solid state NMR analysis of the 1 wt% EGDMA membrane revealed a surprising amount of unreacted vinyl groups still present, see figure 3.6 below
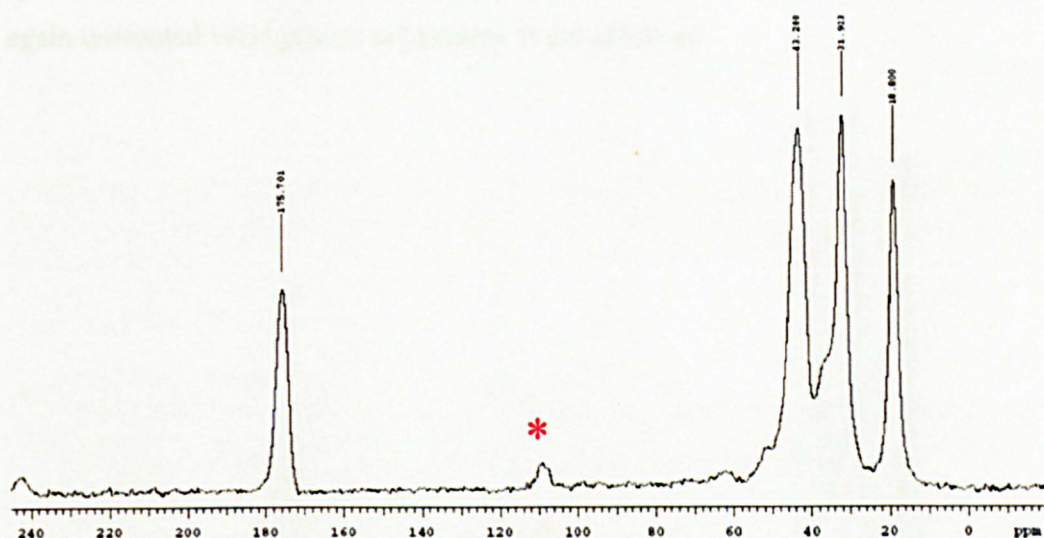

Figure 3.6 ^{13}C NMR spectrum of NVP-co-EGDMA membrane containing 1 wt% EGDMA. The presence of the peak at 110 ppm (*) is indicative of unreacted vinyl groups still present in the polymer membrane.

3.2.2 Synthesis and characterisation of NVP-co-DEGBAC membranes.

The crosslinker was changed to diethyleneglycol bisallylcarbonate (DEGBAC) in an attempt to produce membranes with increased material properties. This crosslinker was also chosen because of the potential for degradation inherent in the carbonate groups. As the aim of this part of the study was the improvement of the material

properties these membranes were synthesised solely in bulk. Increasing the amount of crosslinker in these DEGBAC crosslinked membranes produced clear colourless membranes. The membranes containing 3 wt% DEGBAC and above shattered during the ethanol swelling procedure with the 3 and 4 wt% membranes producing pieces of sufficient size for EWC measurements to be performed. However for those membranes for which EWC measurements were possible the NVP-co-DEGBAC membranes showed reduced water contents compared to NVP-co-EGDMA membranes. The 1 wt% DEGBAC had an EWC of 92.2% ± 0.1% and the EWC reduced with increasing crosslinker concentration. The 2 wt% DEGBAC had an EWC of 89.3% ± 0.2 and 3 wt% DEGBAC had an EWC of 86.1% ± 0.5% and 4 wt% DEGBAC had an EWC of 81.7% ± 0.4%. These are illustrated in table 3.1 and figure 3.4. The 5 wt% DEGBAC membrane shattered into pieces so small that EWC measurements were not possible. Figure 3.7 shows the ^{13}C solid state NMR spectrum from the analysis performed on the 1 wt% DEGBAC membrane, once again unreacted vinyl groups are present in the spectrum.
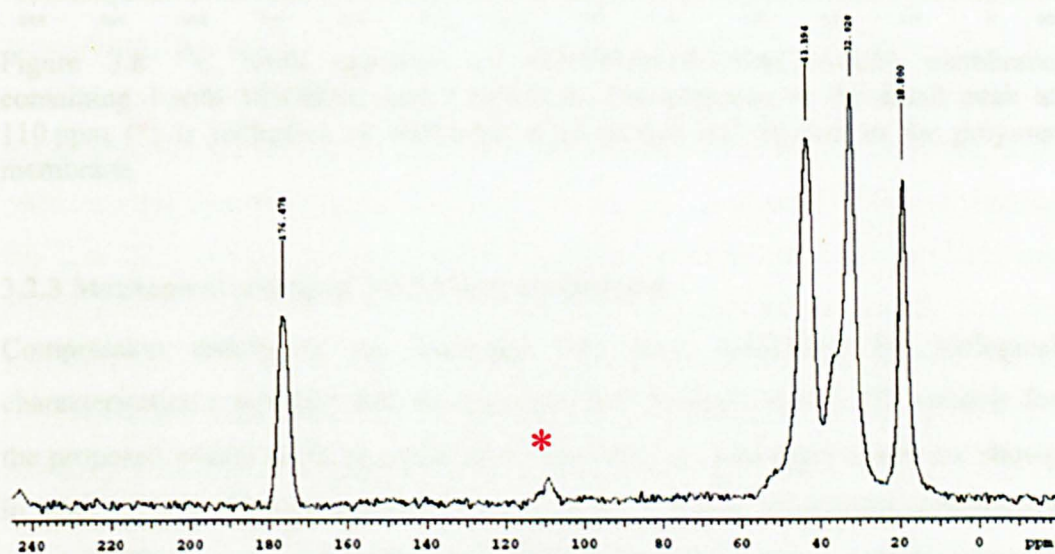

Figure 3.7 ^{13}C NMR spectrum of P(NVP-co-DEGBAC) membrane containing 1 wt% DEGBAC. The presence of the small peak at 110 ppm (*) is indicative of unreacted vinyl groups still present in the polymer membrane.

A small amount (1 wt%) of acrylic acid (AA) was added to the NVP 1 wt% DEGBAC membrane to see if this altered the release profile of either of the anti-contraction agents under investigation see chapter 5. This produced a clear, colourless membrane which became slightly hazy when swollen in PBS indicating a

certain amount of phase separation within the membranes. The addition of the AA reduces the EWC still further. All EWC measurements can be found in table 3.1 and figure 3.4. Again the ^{13}C solid state NMR spectrum in figure 3.8 reveals the presence of unreacted vinyl groups in the membranes.
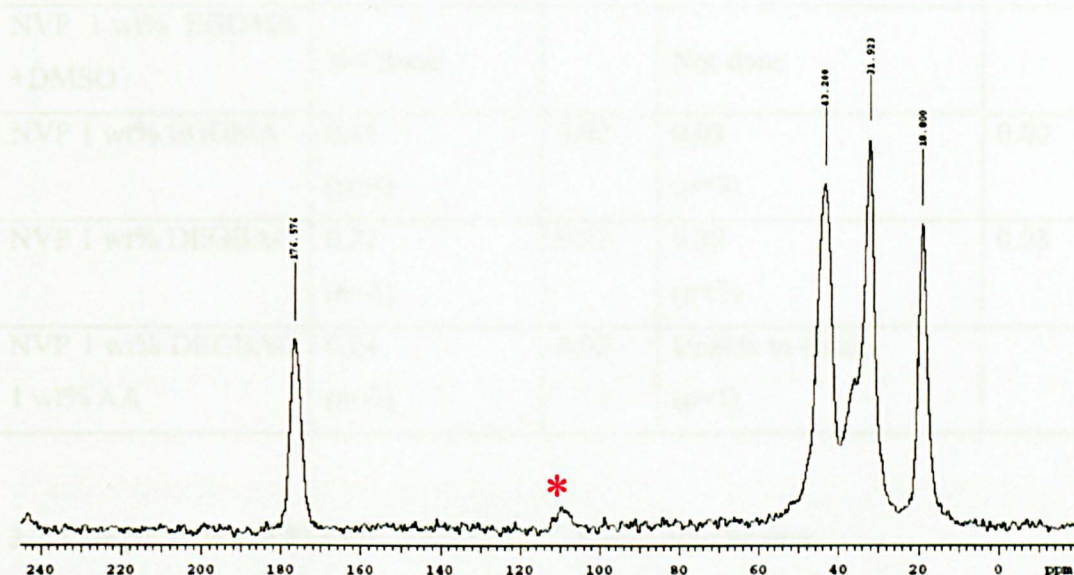

Figure 3.8 ^{13}C NMR spectrum of P(NVP-co-DEGBAC-co-AA) membrane containing 1 wt% DEGBAC and 1 wt% AA. The presence of the small peak at 110 ppm (*) is indicative of unreacted vinyl groups still present in the polymer membrane.

3.2.3 Mechanical testing of NVP based membranes.

Compression testing of the hydrogels that were considered for biological characterisation confirmed that the hydrogels had Young's moduli (E) suitable for the proposed wound dressing application. The data for these experiments are shown in table 3.2. For those samples which were tested to failure an ultimate compressive stress (UCS) was calculated. Some samples did not fail over the 2 mm test distance possibly due to differences in sample thickness. Those that did not fail were excluded from UCS calculations and this lead to different replicate numbers for each polymer.

Table 3.2 Material properties, E and UCS. Measurements performed in compression on polymers swollen in PBS in air over a distance of 2 mm at a strain rate of 0.1 mm.s^{-1}.

Polymer	Elastic Modulus / MPa	SEM / MPa	Ultimate Compressive Stress / MPa	SEM / MPa
NVP 1 wt% EGDMA +DMSO	Not done		Not done	
NVP 1 wt% EGDMA	0.16 (n=4)	0.02	0.03 (n=3)	0.00
NVP 1 wt% DEGBAC	0.22 (n=4)	0.02	0.29 (n=2)	0.08
NVP 1 wt% DEGBAC 1 wt% AA	0.24 (n=3)	0.02	Unable to obtain (n=3)	

3.2.3 Degradation of P(NVP-co-DEGBAC) based membranes

Degradation of P(NVP-co-DEGBAC) hydrogels was initially determined by examining the change in equilibrium water content (EWC), the data is shown in figure 3.9. Previous studies of carbonate crosslinked PNVP's (Bruining, Koole et al. 1999; Liu and Rimmer 2002b) suggest that these polymers should degrade rapidly via carbonate hydrolysis, so initially measurements were taken every 24 hours. However it rapidly became apparent that these polymers did not degrade over the time period studied. The EWC stayed fairly constant over the 10 day period. At this point the number of samples left and difficulties keeping the samples at 37°C meant that the experiment was continued at room temperature. Degradation from this point on was assessed visually, and no apparent degradation was observed after 1 year.
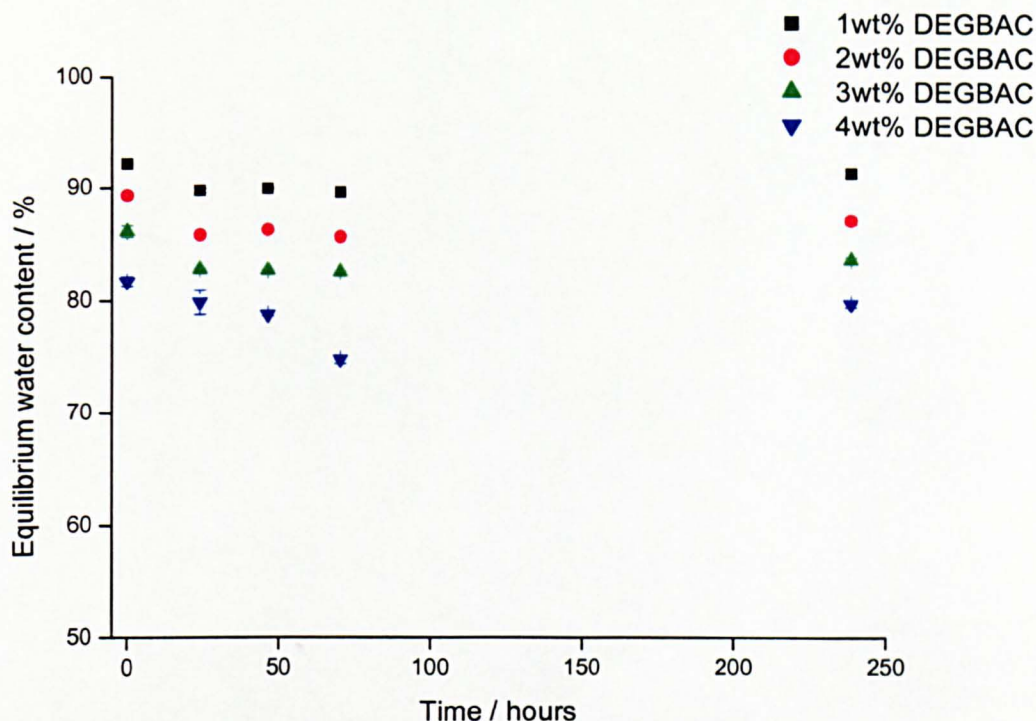
Figure 3.9 Effect of crosslinker concentration on the degradation of P(NVP-co-DEGBAC) hydrogels swollen in PBS at 37°C. Degradation assessed using EWC measurements, measurements expressed as mean ± SEM (n=6).

3.2.4 Synthesis of NVP-co-DEGBAC membranes incorporating βAPN.

In any drug delivery application controlled release is essential. Too little drug and the desired effect is not achieved and too much drug could be harmful to the cells and tissues involved. The incorporation of a known amount of the anti-contraction agents would hopefully allow an appropriate therapeutic concentration to be released. Experiments have shown that βAPN at a concentration of 200 μg.ml^{-1} is effective at reducing contraction of the reconstructed skin model (Chapter 6). Initial experiments were performed attempting to incorporate βAPN, in the form of the water soluble salt 3-aminopropionitrile fumarate (βAPN.F), into the PNVP-co-DEGBAC membranes.

Initially membranes were synthesised where βAPN.F at various concentrations was mixed into the monomer blend before injection into the mould. These membranes were very difficult to peel off the PET sheets. With increasing concentrations of βAPN.F the membranes became increasingly golden in appearance. This was

presumed to be the organic βAPN.F. The βAPN.F had a tendency to locate around the edges of the membrane. A large number of bubbles were seen in the membranes. Due to this and the presence of small βAPN.F crystals still present in the monomer blend prior to injection, a range of solvents was then tested to improve the incorporation of the βAPN.F into the P(NVP-co-DEGBAC) membranes.

Table 3.3 Table showing the solubility, assessed visually, of 3-aminopropionitrile fumarate in 1 ml of solvent.

Solvent	Solubility	Solvent	Solubility
10% EtOH in DMSO	Insoluble	DMF	Insoluble
20% EtOH in DMSO	Insoluble	Dioxane	Insoluble
30% EtOH in DMSO	Insoluble	THF	Insoluble
40% EtOH in DMSO	Insoluble	IPA	Insoluble
50% EtOH in DMSO	Insoluble	Acetonitrile	Insoluble
60% EtOH in DMSO	Insoluble	DEGBAC	Insoluble
70% EtOH in DMSO	Insoluble	Methanol	Insoluble
80% EtOH in DMSO	Insoluble	AA	Soluble
90% EtOH in DMSO	Insoluble	NVP	Insoluble
10% H$_2$O in NVP	Insoluble	17% H$_2$O in NVP	Insoluble
15% H$_2$O in NVP	Insoluble	20% H$_2$O in NVP	Soluble

With βAPN.F soluble in water a small amount of water (1 ml per membrane) was added to the monomer blend prior to injection. When these membranes still proved unsuitable 20 wt% water was added to the monomer blend. Acrylic acid was also added to the monomer blend.

These membranes still had a golden tinge especially at high concentrations of βAPN.F and a large amount of bubbles were still visible in the membranes. The membranes containing 20 wt% water were easy to peel off the PET backing sheets and extremely pliable. The addition of Acrylic acid to the P(NVP-co-DEGBAC) membranes resulted in bubbles and localisation of the βAPN.F around the edges of the membrane. It was then thought that βAPN could be loaded into dried hydrogel membranes (xerogels) from a known concentration in phosphate buffered saline see chapter 7.

3.2.4 Summary of results

The synthesis of poly(vinylpyrrolidinone) based hydrogels membranes using a mixture of 1-vinyl-2-pyrrolidinone, crosslinker and initiator ± solvent was successful and produced membranes with material properties suitable for use as a wound dressing. Changing the crosslinker from EGDMA to DEGBAC and removing solvent from the reaction reduced the water content sufficiently to produce a tough flexible hydrogel. The addition of acrylic acid to the P(NVP-co-DEGBAC) membranes reduced the EWC still further with a corresponding increase in Young's modulus. Young's moduli were achieved of between 0.16 MPa ± 0.02 MPa and 0.24 MPa ± 0.02 MPa when tested at a strain rate of 0.1 mm.s^{-1} (6 mm.min^{-1}) for the polymers synthesised *in* bulk. These values compare favourably with those seen in the literature. Cauich-Rodriguez et al. (1996a) synthesised a poly(vinylalcohol) – poly(vinylpyrrolidinone) blend with similar material properties. The Young's moduli were dependent on the heat treatment received by the polymer but were between 0.15 MPa ± 0.07 MPa and 1.51 MPa ± 1.00 MPa at a strain rate of 5 mm.min^{-1}. The addition of gluteraldehyde to these polymers had no effect on the Young's modulus of these polymers (Cauich-Rodriguez, Deb et al. 1996b). The introduction of carbonate groups into the membranes via the change in crosslinker from EGDMA to DEGBAC did not produce the expected highly degradable hydrogel. A small amount of unreacted vinyl groups could still be found in the ^{13}C solid state NMR spectra of all the hydrogels.

Synthesising P(NVP-co-DEGBAC) membranes where βAPN.F was incorporated into the monomer blend prior to injection into the polymerisation mould proved to be unsuccessful due to the insolubility of βAPN.F in NVP and the other components of the monomer mix. A large amount of water was needed to ensure that the βAPN.F remained dissolved in the monomer blend. However in all the membranes where βAPN.F was added the βAPN.F had a tendency to localise around the edges of the membrane and an unacceptable amount of bubbles were formed during curing. Even the addition of acrylic acid to the membranes did not solve this problem.

4. Effect of Poly(vinylpyrrolidinone) membranes on cell viability.

Experiments in this section were performed with the cells in direct contact with the polymers and also in indirect contact. The poor material properties of the P(NVP-co-EGDMA) polymers meant that to force contact between the polymer and the cells the polymers had to be placed inside ThinCert™ inserts. The commonly used method of seeding cells inside a stainless steel ring did not work in this situation as the weight of the ring cut straight through the P(NVP-co-EGDMA) polymer.

The P(NVP-co-DEGBAC) and P(NVP-co-DEGBAC-co-AA) polymers are stiff enough to be able to support the stainless steel ring. Initial experiments were performed to investigate if cells could attach to the P(NVP-co-DEGBAC) polymers however problems with seepage from the rings due to the smoothness of the polymer surfaces made these experiments difficult to perform and so they were abandoned early on.

4.1 Effect of Poly(vinylpyrrolidinone) membranes on cell viability – direct contact versus indirect contact.

4.1.1 Cytotoxicity of P(NVP-co-EGDMA) membranes.

Initial experiments were performed where P(NVP-co-EGDMA) hydrogels were placed in direct contact with human dermal fibroblasts in the presence of serum. These experiments are summarised in figure 4.2 below. Student's unpaired T-Test was used to determine significant ($p < 0.05$) stimulation or inhibition in cell viability as assessed using the MTT-ESTA assay. A large number of experiments were required to get a clear picture due to the variation in viabilities encountered during these initial experiments. The fibroblasts used were from different donors and different passage numbers and the P(NVP-co-EGDMA) membranes came from multiple batches. None of these variables affected the range of results obtained.
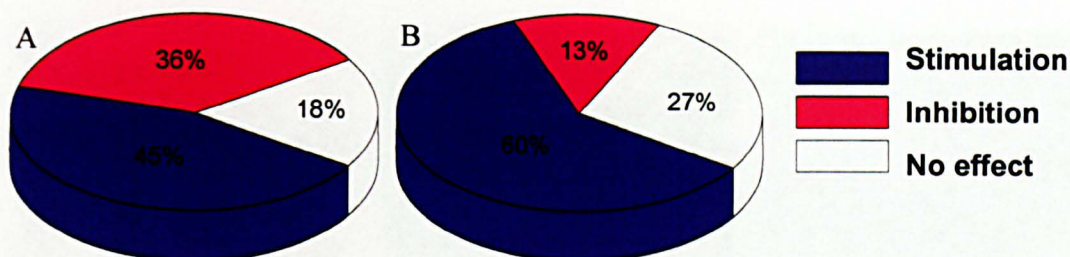
Figure 4.2 Pie charts illustrating the percentage of experiments where cell viability was stimulated, inhibited or not affected by A) Direct contact or B) Indirect contact with P(NVP-co-EGDMA) hydrogels. Stimulation or inhibition represented a statistically significant ($p < 0.05$) difference in cell viability from tissue culture plastic control n=20 (3 replicates per experiment).

The results obtained from cells cultured in direct contact with the polymer can be viewed with some suspicion. It can be seen clearly in figure 4.3 how the cells (stained blue with MTT in this picture) are found at the bottom of the inserts (A) and at the interfaces between the pieces of polymer and the polymer-insert interface. These clumps of cells are viable but do not look normal when examined visually as shown in figure 4.4.A. However fibroblasts grown in indirect contact with the hydrogel have a normal morphology comparable to fibroblasts grown on TCP alone as shown in figure 4.4.C.
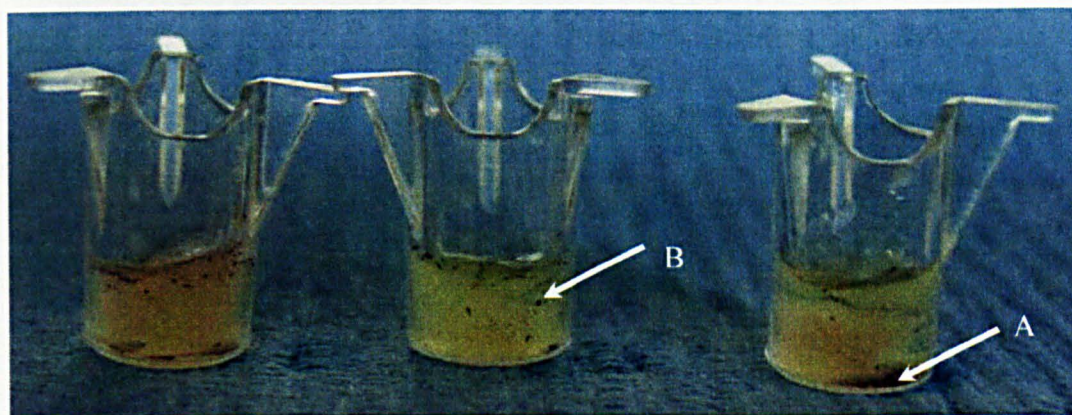

Figure 4.3 Photograph of human dermal fibroblasts cultured in direct contact with P(NVP-co-EGDMA) for 4 days in ThinCert™ inserts in the presence of serum. Cells are stained purple with MTT and can clearly be seen on the bottom of the Insert (A) and in small clumps at the interface between the polymer and insert (B).

Figure 4.4 Photograph of human dermal fibroblasts cultured in the presence of serum for 4 days A) in direct contact with the P(NVP-co-EGDMA) membrane in ThinCert™ inserts B) grown on TCP in indirect contact with the P(NVP-co-EGDMA) membrane and C) Photograph of fibroblasts grown on TCP only. In all photographs cells are stained purple with MTT

4.1.2 Cytotoxicity of P(NVP-co-DEGBAC) membranes.

Poor mechanical properties (see chapter 3) meant that the P(NVP-co-EGDMA) polymer would be unsuitable for the final application of a wound dressing and so another crosslinking agent diethyleneglycol bisallylcarbonate (DEGBAC) was investigated. Again cytotoxicity studies were performed with the polymer in both direct and indirect contact with human dermal fibroblasts in media containing 10% FCS. These experiments are summarised in the pie charts in figure 4.5. As in figure 4.2 Student's unpaired T-Test was used to determine significant ($p < 0.05$) stimulation or inhibition in cell viability as assessed using the MTT-ESTA assay.
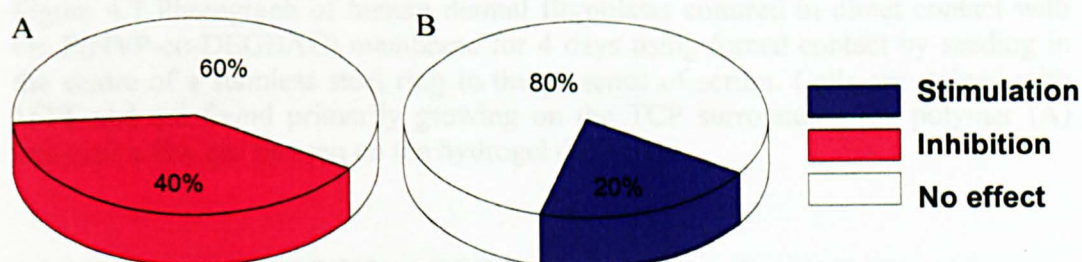

Figure 4.5 Pie charts illustrating the percentage of experiments where cell viability was stimulated, inhibited or not affected by A) Direct contact or B) Indirect contact with P(NVP-co-DEGBAC) hydrogels. Stimulation or inhibition was determined within each experiment by a statistically significant ($p < 0.05$) difference in cell viability from TCP control n=5 (3 replicates per experiment).

Once again the cells are found around the edges of the polymer and underneath the polymer growing on the PET membrane on the bottom of the insert. Cells that have attached to the membrane, see figures 4.6 and 4.7, again do not look normal however

these cells have wetted to the surface and are less rounded than those attached to the P(NVP-co-EGDMA) membrane.
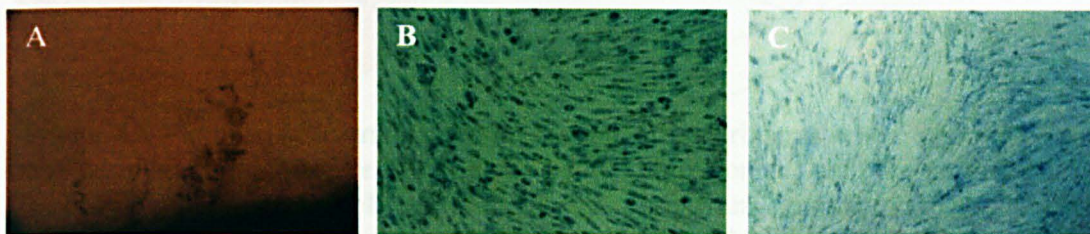

Figure 4.6 Photograph of human dermal fibroblasts cultured in the presence of serum for 4 days A) in direct contact with the P(NVP-co-DEGBAC) membrane in ThinCert™ inserts B) grown on tissue culture polystyrene in indirect contact with the P(NVP-co-DEGBAC) membrane and C) Photograph of fibroblasts grown on tissue culture polystyrene only. In all photographs cells are stained purple with MTT.
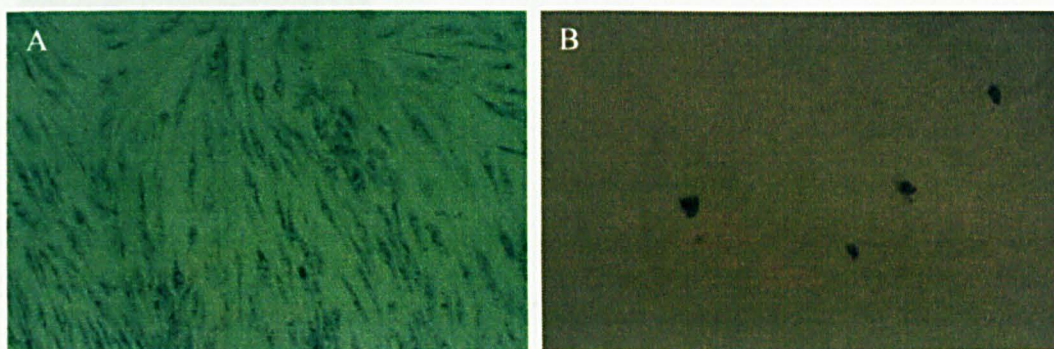

Figure 4.7 Photograph of human dermal fibroblasts cultured in direct contact with the P(NVP-co-DEGBAC) membrane for 4 days using forced contact by seeding in the centre of a stainless steel ring in the presence of serum. Cells are stained with MTT and are found primarily growing on the TCP surrounding the polymer (A) however a few can be seen on the hydrogel (B).

4.1.3 Cytotoxicity of P(NVP-co-DEGBAC-co-AA) membranes.

1 wt% acrylic acid (AA) was subsequently incorporated into the P(NVP-co-DEGBAC) membranes in an attempt to modify the release of βAPN from the polymers see chapter 7. Cytotoxicity studies performed on these polymers where human dermal fibroblast cells were placed in direct and indirect contact with the polymer in both the presence and absence of serum are summarised in figure 4.8. Fibroblasts cultured in direct contact with the polymers P(NVP-co-DEGBAC-co-AA) polymers once again preferred to grow on the PET insert membrane than on the hydrogels. Figure 4.9 shows human dermal fibroblasts stained with MTT growing on the insert instead of on the P(NVP-co-DEGBAC-co-AA) hydrogels.
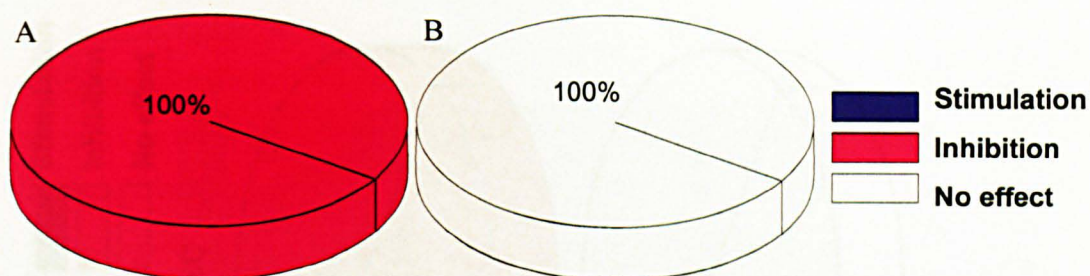
Figure 4.8 Pie charts illustrating the percentage of experiments where cell viability was stimulated, inhibited or not affected by A) direct contact with the polymer, B) indirect contact with the polymer in the presence of serum, stimulation or inhibition was taken to mean a statistically significant ($p<0.05$) difference in cell viability from TCP control within each experiment n=3 (3 repeats per experiment)
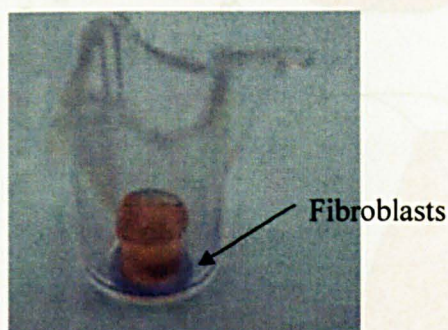

Figure 4.9 Photograph of human dermal fibroblasts cultured in direct contact with P(NVP-co-DEGBAC-co-AA) for 4 days in ThinCert^{TM} inserts in the presence of serum. Cells are stained purple with MTT and can clearly be seen on the bottom of the insert.

4.1.4 Cytotoxicity of P(NVP-co-DEGBAC) membranes on a variety of skin and epithelial cells.

Whilst fibroblasts are suitable for generic cytotoxicity testing they are by no means the only cell type found in skin. These polymers will hopefully be used in a wound healing application; therefore the polymers will primarily come into contact with keratinocytes. Primary normal human keratinocytes and a keratinocyte cell line, the HaCaT cell line along with a human cornea epithelial cell line (HCEC) were cultured in optimised medium for each cell type in direct and indirect contact with the P(NVP-co-DEGBAC) polymer. The results are shown in table 4.1 with photomicrographs of cell morphology shown in table 4.2.

Table 4.1 Effect of PNVP on viability of HaCaT, HECE and Keratinocyte cells. Cells were grown in direct and indirect contact with P(NVP-co-DEGBAC) for 4 days. Results shown are the means ± SEM of 3 experiments (3 replicates per experiment). *p< 0.05, **p< 0.01, ***p< 0.001
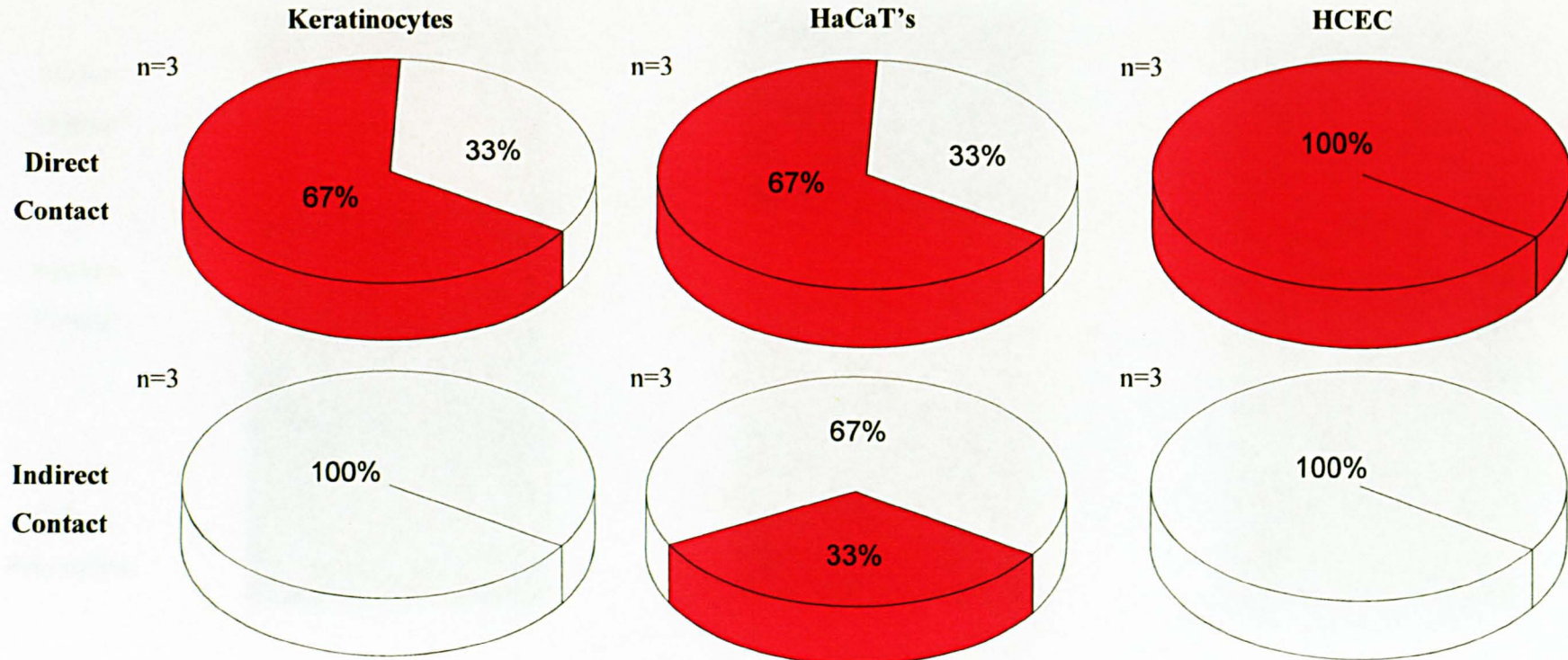
Table 4.2 photographs of primary normal human keratinocytes, HaCaT's and human cornea epithelial cells stained with MTT after being grown in direct or indirect contact with P(NVP-co-DEGBAC) and on tissue culture polystyrene with no P(NVP-co-DEGBAC) present in optimised medium.

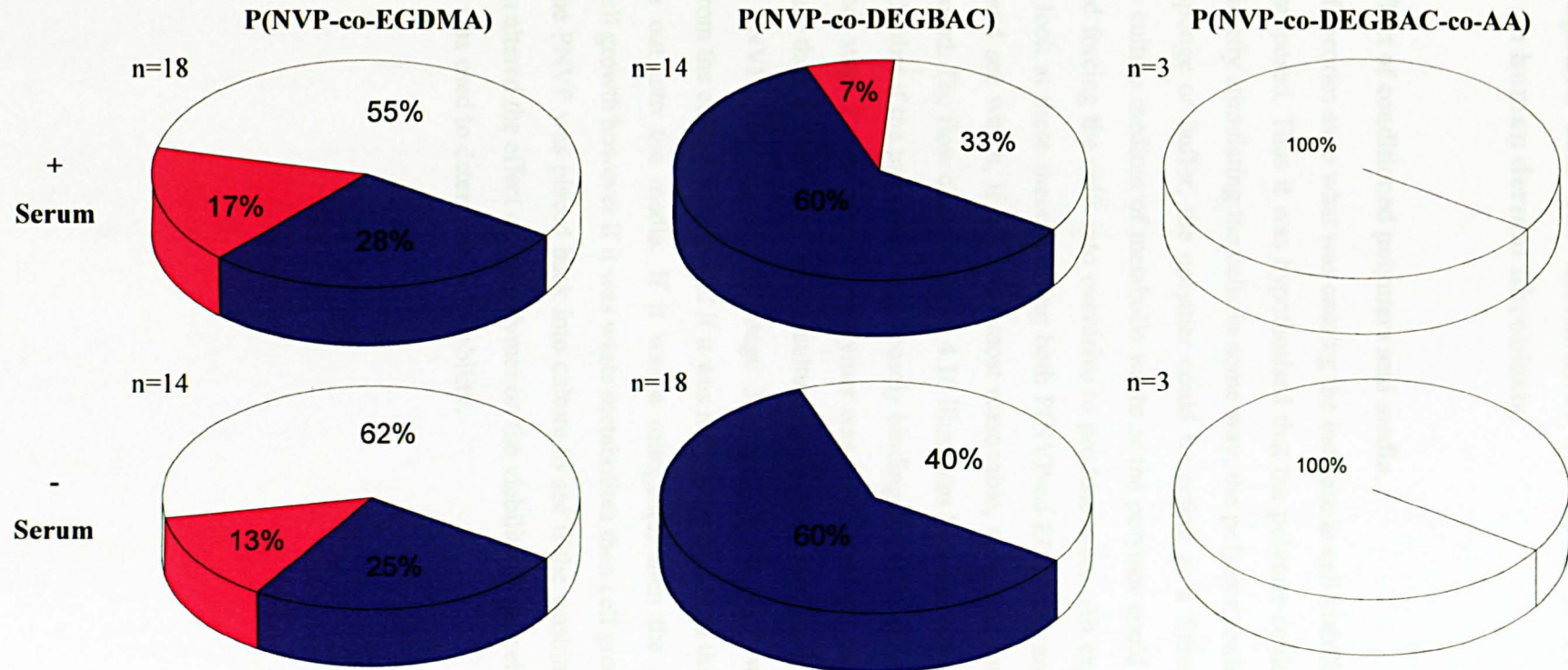|	Keratinocytes	HaCaT's	HCEC
Direct Contact			
Indirect Contact			
Tissue Culture Polystyrene			
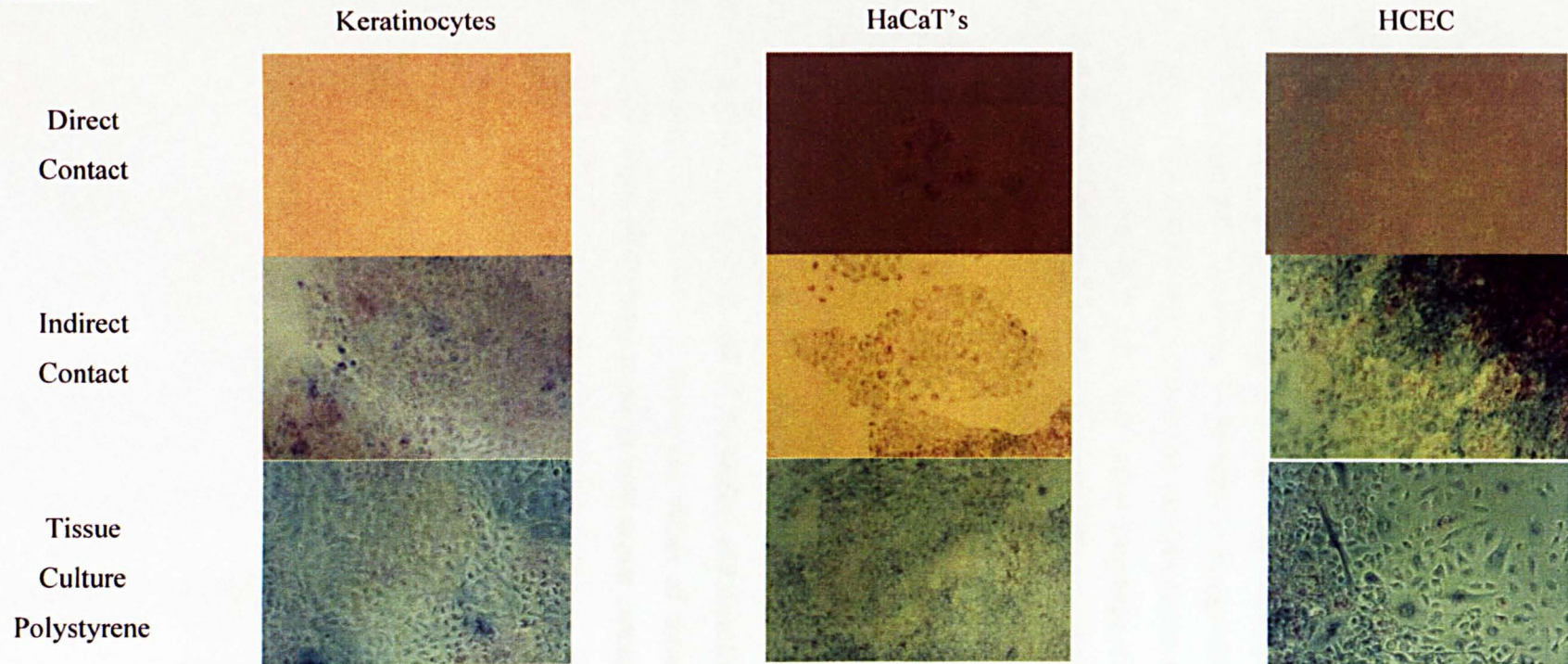
4.2 Effect of PNVP membranes on cell viability – The effect of serum.

The ability to successfully culture cells in serum free conditions is an ambition of many laboratories due to fears over the disease transmission between species i.e. bovine spongioform encephalitis (BSE) and its human analogue Creutzfeld Jacob Disease (CJD). Hong et al. (1997) showed that poly(vinylpyrrolidinone) hydrogels crosslinked with diethyleneglycol dimethacrylate (DEGDMA) could increase the viability of 3T3 cells cultured in serum free conditions to approximately 90% of their viability in cultures containing serum. However the crosslinker used in the polymer synthesis was shown to moderate this with some crosslinkers proving toxic. The indirect contact system was used to see if the crosslinkers used in these experiments, i.e. EGDMA, DEGBAC and DEGBAC + AA had a similar effect.

Initial experiments were performed using the P(NVP-co-EGDMA) and P(NVP-co-DEGBAC) polymers hence for these polymers there are many more experiments for analysis than with the P(NVP-co-DEGBAC-co-AA) hydrogels which were developed at a much later stage in this project. The latter were developed to modify the release of βAPN.F towards the end of the project and therefore there were fewer of these for examination. Table 4.3 shows the effect of these hydrogels on the viability of human dermal fibroblasts grown in both serum containing and serum free medium.

Table 4.3 Effect of PNVP membranes with different crosslinkers on the viability of human dermal fibroblasts in the presence and absence of 10% foetal calf serum in the culture media. Experiments were performed using the indirect contact culture system. Stimulation or inhibition was determined by a statistically significant ($p<0.05$) difference in cell viability compared to the same cells grown on tissue culture plastic. (3 repeats per experiment)

Legend:
- Stimulation
- Inhibition
- No effect

P(NVP-co-EGDMA)

+ Serum: n=18 — 55%, 17%, 28%

– Serum: n=14 — 62%, 13%, 25%

P(NVP-co-DEGBAC)

+ Serum: n=14 — 7%, 33%, 60%

– Serum: n=18 — 40%, 60%

P(NVP-co-DEGBAC-co-AA)

+ Serum: n=3 — 100%

– Serum: n=3 — 100%

4.3 Investigation into the mechanism of action of PNVP based hydrogels on human dermal fibroblasts.

4.3.1 The effect of conditioned polymers and media.

A number of theories as to what was causing the increase in cell viability had by this point been proposed. Thus it was hypothesised that the polymer could be breaking down and directly stimulating the cells in some way, the polymer could be acting as a mitogen sponge or buffer, the polymer could be acting as a filtration unit and cleaning the culture medium of metabolic waste or the polymer could be collecting mitogens and forcing the cells into overdrive to produce more. An experiment was designed to look at these theories using both P(NVP-co-EGDMA) and P(NVP-co-DEGBAC) and see which, if any, was most reasonable, again the indirect contact system was used. The flow chart in figure 4.10 illustrates how the experiment works. It was thought that if the polymers were loosely binding something during the initial culture period, stage 1, then when the polymer was placed straight back above new cells in stage 2 then the action of the pre-cultured polymer would be different to that of the new PNVP. The premise for stage 3 is that if the NVP was removing something from the culture system and if it was not tightly bound to the NVP then it would wash out into the media. If it was a mitogen(s) then the media would encourage cell growth however if it was waste metabolites then cell growth would be inhibited. The PNVP was placed back into culture to see if the washing step during stage 2 again altered the effect of the polymer on the viability of the cells. The MTT-ESTA assay was used to determine cell viability.
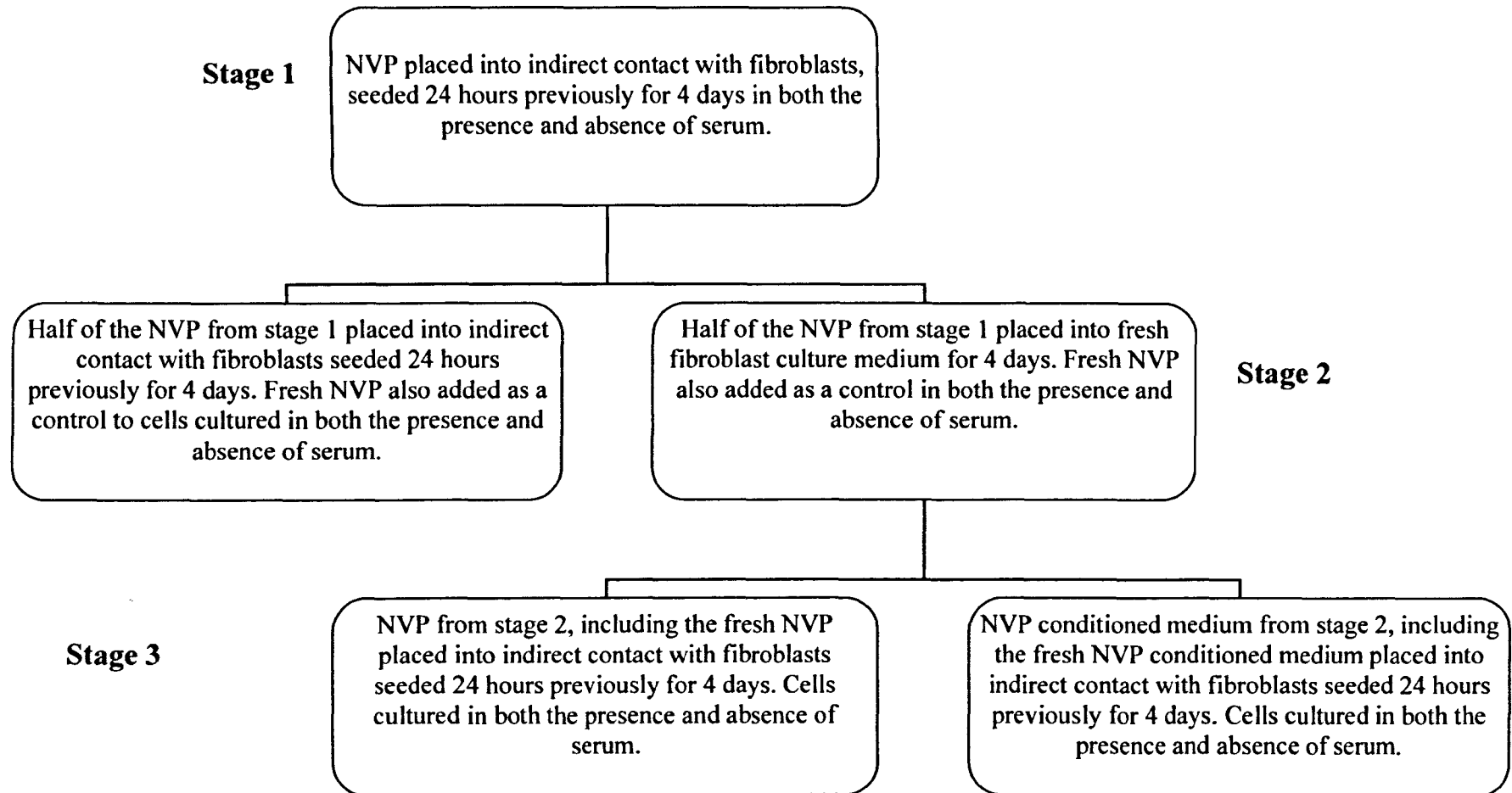
Stage 1

NVP placed into indirect contact with fibroblasts, seeded 24 hours previously for 4 days in both the presence and absence of serum.

Half of the NVP from stage 1 placed into indirect contact with fibroblasts seeded 24 hours previously for 4 days. Fresh NVP also added as a control to cells cultured in both the presence and absence of serum.

Half of the NVP from stage 1 placed into fresh fibroblast culture medium for 4 days. Fresh NVP also added as a control in both the presence and absence of serum.

Stage 2

Stage 3

NVP from stage 2, including the fresh NVP placed into indirect contact with fibroblasts seeded 24 hours previously for 4 days. Cells cultured in both the presence and absence of serum.

NVP conditioned medium from stage 2, including the fresh NVP conditioned medium placed into indirect contact with fibroblasts seeded 24 hours previously for 4 days. Cells cultured in both the presence and absence of serum.

Figure 4.10 Flow chart illustrating the organisation of the experiment designed to investigate how the PNVP polymers were affecting cell viability.

Stage 1 of this experiment shown in figure 4.11 illustrates the effect that P(NVP-co-EGDMA) and P(NVP-co-DEGBAC) have on fibroblast viability using the indirect contact method assessed using the MTT-ESTA assay. Cells were grown in both the presence and absence of serum. Only the P(NVP-co-EGDMA) hydrogel significantly increased fibroblast viability in these three experiments and this was only in the presence of serum.
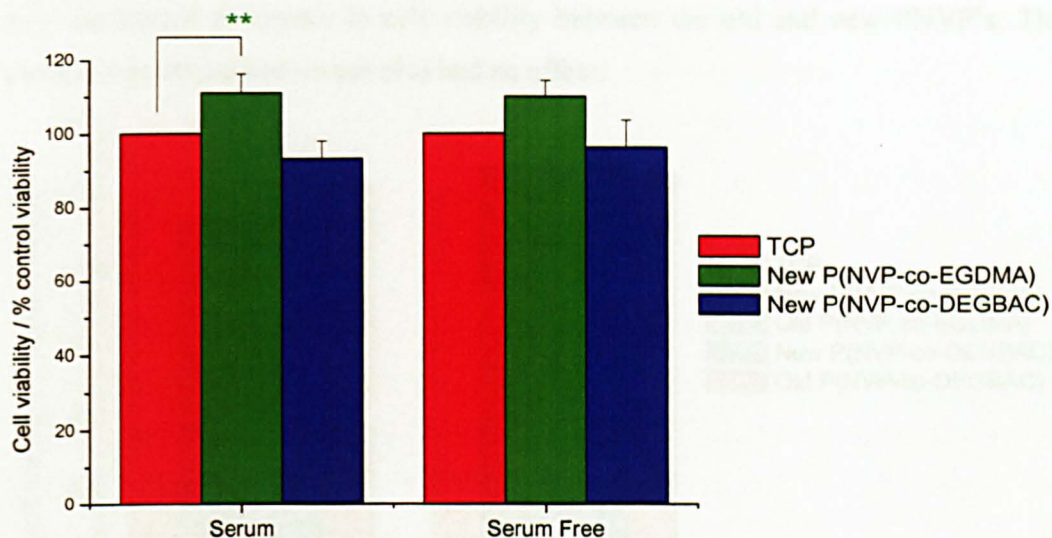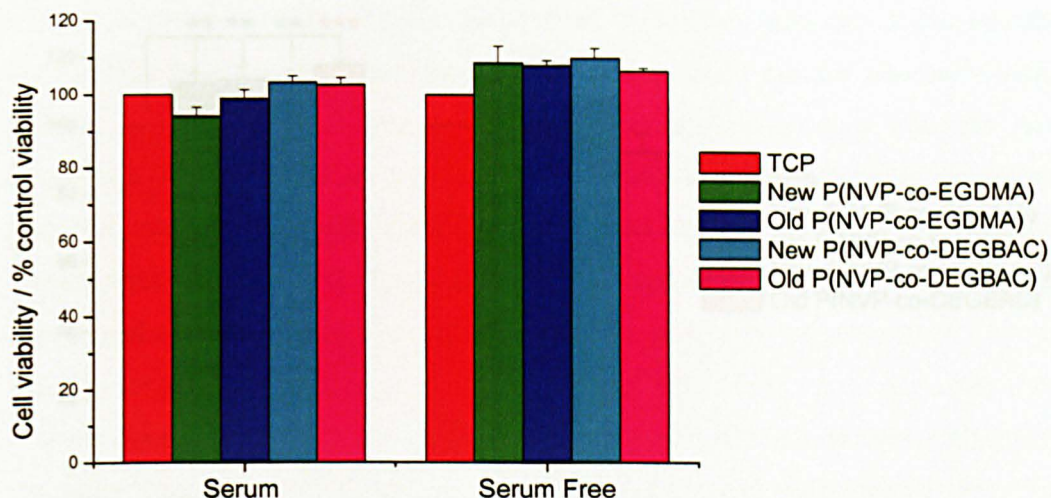

Figure 4.11 Effect of PNVP on fibroblast viability. Cells were grown in indirect contact with PNVP for 4 days in the presence and absence of serum. Results shown are the means ± SEM of 3 experiments (3 replicates per experiment). Significance was assessed using Student's T-Test *p< 0.05, **p< 0.01, ***p< 0.001.

Stage 2 of this experiment shown in figure 4.12 illustrates the effect that P(NVP-co-EGDMA) and P(NVP-co-DEGBAC) polymers that have previously been in indirect contact with fibroblasts for 4 days have on the viability of a new set of fibroblasts. The experiment was again performed in both the presence and absence of serum. Fresh hydrogels kept in fibroblast culture medium, either in the presence or absence of serum were also present. At no point during this part of the experiment was there any significant difference in cell viability between the old and new PNVP's. The presence or absence of serum also had no effect.

Figure 4.12 Effect of PNVP on fibroblast viability. Cells were grown in indirect contact with PNVP for 4 days in the presence and absence of serum. Results shown are the means ± SEM of 3 experiments (3 replicates per experiment). Significance was assessed using Student's T-Test *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Stage 3 of this experiment shown in figure 4.13 illustrates the effect that P(NVP-co-EGDMA) and P(NVP-co-DEGBAC) polymers that have previously been in indirect contact with fibroblasts for 4 days and subsequently washed for 4 days, have on the viability of a new set of fibroblasts. The experiment was again performed in both the presence and absence of serum. Cell viability was significantly increased when the washed polymers were placed in indirect contact with cells in the presence of serum. These polymers had no significant effect on cell viability in the absence of serum. Again there is no difference between the old and new polymers.
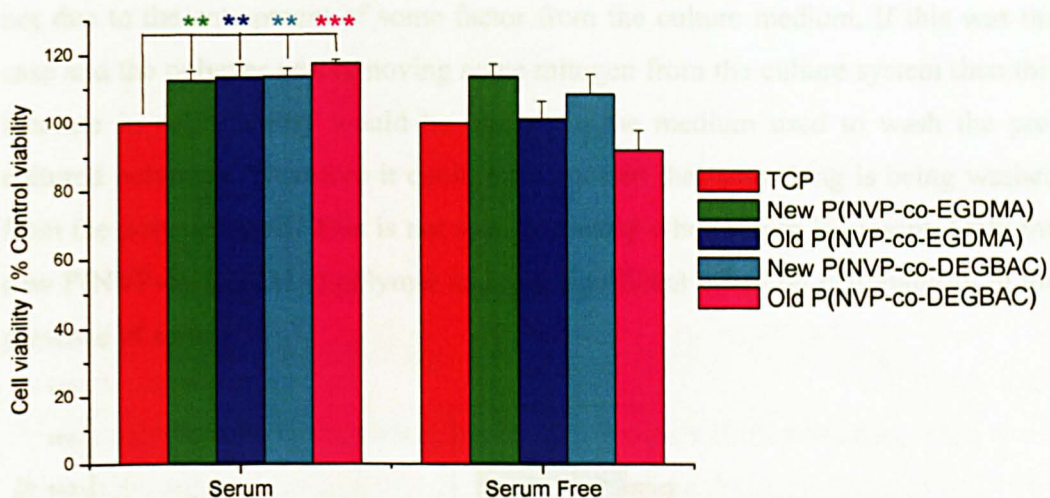

Figure 4.13 Effect of PNVP on fibroblast viability. Cells were grown in indirect contact with washed PNVP for 4 days in the presence and absence of serum. Results shown are the means ± SEM of 3 experiments (3 replicates per experiment). Significance was assessed using Student's T-Test *p< 0.05, **p< 0.01, ***p< 0.001.

Stage 3 of this experiment shown in figure 4.14 illustrates the effect that medium used to wash P(NVP-co-EGDMA) and P(NVP-co-DEGBAC) polymers that have previously been in indirect contact with fibroblasts for 4 days and medium used to wash fresh PNVP have on the viability of a new set of fibroblasts. The experiment was again performed in both the presence and absence of serum. In all cases when the polymers have been placed into serum free medium the cell viability is increased. There is no difference between the media used to wash the new polymers and the pre-cultured polymers suggesting that the effect of the polymers on cell viability is not due to the entrapment of some factor from the culture medium. If this was the case and the polymer was removing some mitogen from the culture system then this increase in cell viability would be greater in the medium used to wash the pre-cultured polymers. Therefore it could be suggested that something is being washed from the polymer itself. This is not seen as clearly when serum is present. Only the new P(NVP-co-EGDMA) polymer had any significant effect on cell viability in the presence of serum.
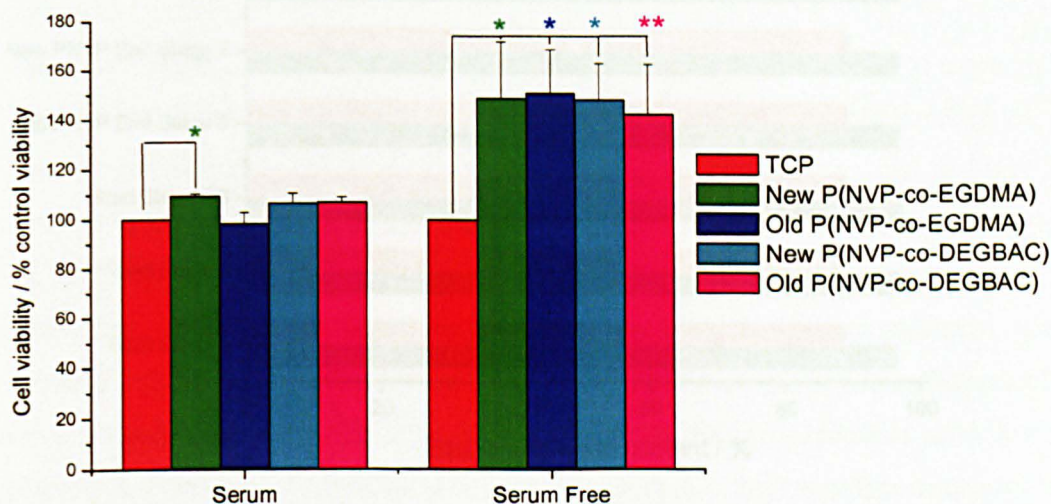

Figure 4.14 Effect of PNVP conditioned medium on fibroblast viability. Cells were grown in indirect contact with PNVP conditioned medium for 4 days in the presence and absence of serum. Results shown are the means ± SEM of 3 experiments (3 replicates per experiment). Significance was assessed using Student's T-Test *p< 0.05, **p< 0.01, ***p< 0.001.

Throughout the experiment the equilibrium water content of the samples was monitored. The equilibrium water contents of the hydrogels were measured at the start and end of each stage. These hydrogels were treated exactly the same way as the hydrogels used for the cell culture part of this experiment above. However at no time did these hydrogels come into contact with cells. Each piece of hydrogel was placed into 1ml of fibroblast culture medium, both in the presence and absence of serum and kept in an incubator at 37°C for the same length of time as the hydrogels placed into indirect contact with the fibroblasts. At no time over the 12 days of the experiments was any significant difference in equilibrium water content observed within any of the stages of the experiment with the results shown in figure 4.15 below.
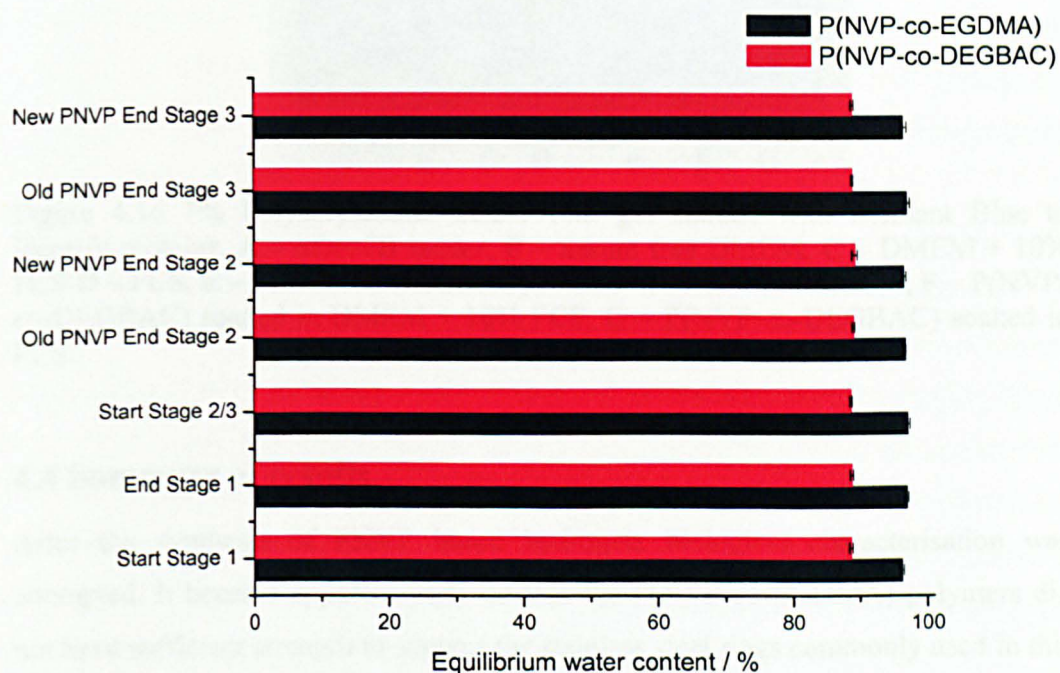

Figure 4.15 Effect of pre-culture and washing of the polymer on the equilibrium water content of P(NVP-co-EGDMA) and P(NVP-co-DEGBAC) hydrogels. Results expressed as mean ± SEM of n=6 samples. There was no significant difference in the equilibrium water contents for each polymer observed over the course over the 12 days of the experiment.

4.3.2 Investigation into protein binding to PNVP hydrogels.

A possible mechanism by which the PNVP hydrogels could affect the viability of fibroblasts would be through the binding and / or release of proteins and growth factors. SDS-PAGE electrophoresis with subsequent protein staining with Brilliant Blue [TM] was used to briefly investigate this possibility.
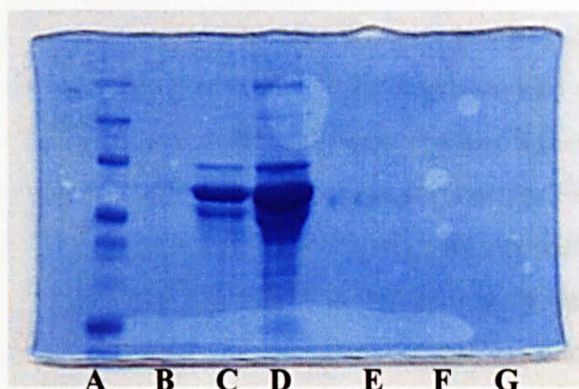

Figure 4.16 7% Polyacrylamide SDS-PAGE gel stained with Brilliant Blue to identify proteins. A – SDS-7B ladder, B – Serum free DMEM, C – DMEM + 10% FCS D – FCS, E – P(NVP-co-DEGBAC) soaked in Serum free DMEM, F – P(NVP-co-DEGBAC) soaked in DMEM + 10% FCS, G – P(NVP-co-DEGBAC) soaked in FCS.

4.4 Summary of results.

After the synthesis of PNVP based hydrogels biological characterisation was attempted. It became apparent early on that the P(NVP-co-EGDMA) polymers did not have sufficient strength to support the stainless steel rings commonly used in this laboratory to force contact between cells and substrates. It was therefore necessary to develop an alternate methodology to force this contact. The use of ThinCert[TM] inserts was determined to be the best way forward, as this system also gave the opportunity to examine the effect of these polymers when placed in indirect contact with cells.

Examination of the effects of PNVP based polymers synthesised for this study on the viability of human dermal fibroblasts showed that it was difficult to grow cells in direct contact with the polymers. Those cells that did manage to attach did not appear when viewed down a microscope to be viable. Those cells that did manage to

escape from direct contact with the polymers preferred to grow elsewhere i.e. the tissue culture plastic on the bottom of the wells or the PET membrane on the bottom of the ThinCert™ inserts. This could explain the discrepancy between the MTT-ESTA viability data and the appearance of the cells when grown in direct contact with the polymers. The lack of cell adhesion to these polymers can perhaps be explained by the extremely high water contents of these polymers. The high hydrophilicity would make protein adsorption to the surfaces extremely difficult and the lack of adsorbed proteins would decrease the sites available for cell attachment. Haigh et al. (2002) showed that the EWC of hydrogels had an effect on fibroblast adhesion and the state of the water in the hydrogels was also a factor. Hydrogels with an EWC of 30% appeared to have the optimum conditions for fibroblast attachment and spreading. Levels of attachment were lower on polymers with a EWC of greater than 30%. Also the presence of free water in the hydrogels was only observed at EWC's greater than 25% and this was necessary for cell attachment and spreading (Haigh, Fullwood et al. 2002).

In nearly all cases where fibroblasts were cultured in indirect contact with the hydrogels the viability, assessed using the MTT-ESTA assay, was either unaffected by the presence of the polymers or increased. This correlates with the visual analysis of cell morphology. The fibroblast viability when grown in indirect contact with the PNVP based polymers ranged from 46 - 530% of control viability in the presence of serum and 15 - 242% of control viability without serum. The average increase in viability was 22.5% $\pm$ 24.6% for the P(NVP-co-EGDMA) polymer in the presence of serum (n=18 not significant, Students paired T-Test) and 27.2% $\pm$ 16.6% in the absence of serum (n=14 not significant, Students paired T-Test), overall the P(NVP-co-EGDMA) polymer increased the average viability by 24.5% $\pm$ 15.4% regardless of the presence or absence of serum. The P(NVP-co-DEGBAC) polymer increased the average viability of fibroblasts grown in indirect contact with the polymer by 19.5% $\pm$ 11.8% in the presence of serum (n=14 $p<0.05$, Students paired T-Test) and 19.2% $\pm$ 19.2% in the absence of serum (n=18 $p<0.01$, Students paired T-Test) and regardless of the presence or absence of serum the average increase in cell viability was 19.4% $\pm$ 7.0%. Overall PNVP based hydrogels increased cell viability by 22.1% $\pm$ 8.5% regardless of the presence of serum and crosslinker used (n=64 $p<0.01$, Students paired T-Test). PNVP hydrogels increased the average cell viability by

21.2% ± 14.6% in the presence of serum and 23.1% ± 8.8% in the absence of serum. The P(NVP-co-DEGBAC-co-AA) hydrogels had no significant effect on cell viability in both the presence and absence of serum when human dermal fibroblasts were grown in indirect contact with the polymer.

Attempts were made to grow primary human keratinocytes, the HaCaT keratinocyte cell line and a human cornea epithelial cell line (HCEC) in both direct and indirect contact with the P(NVP-co-DEGBAC) polymer. The MTT-ESTA assay was again used to assess cell viability along with visual observation of cell morphology. In all cases when the cells were grown in direct contact cell viability was decreased. HCEC cells and keratinocytes cannot be grown successfully on tissue culture plastic (TCP) alone. For successful culture HCEC cells need to be grown on TCP coated with a mixture of extracellular matrix proteins (collagen I, bovine serum albumen and fibronectin). Keratinocytes need to be grown on TCP coated with collagen I. HaCaT cells can be grown successfully on uncoated TCP. The P(NVP-co-DEGBAC) had no significant effect on the viability of keratinocytes and HCEC cells grown for 4 days in indirect contact with the polymer in all three experiments. In two out of the three experiments the P(NVP-co-DEGBAC) hydrogel had no significant effect on the viability of HaCaT cells grown in indirect contact with the polymer. In one out of the three experiments where HaCaT cells were grown in indirect contact with the P(NVP-co-DEGBAC) hydrogel a significant decrease in cell viability was observed.

Attempts to explain how these hydrogels affected the viability of human dermal fibroblasts involved investigating the action of both conditioned polymers and polymer conditioned media. The polymer could be breaking down. The polymer could be removing mitogens or toxins and metabolic waste from the media. These could either be released later or removed from the system completely, forcing the cells into overdrive. These results showed that polymers once placed in indirect contact with fibroblasts for 4 days and then immediately placed into indirect contact with another batch of fibroblasts had no significant effect on the viability of this second batch of fibroblasts in both the presence and absence of serum. However if these polymers were washed before being placed back into indirect contact with the fibroblasts in the presence of serum an increase in viability was seen, but there was

no significant difference between the old and new PNVP's. In the absence of serum there was no significant difference between the cells cultured in the presence or absence of PNVP or between the old and new PNVP's. The conditioned media from the old PNVP's also had no significant effect on cell viability in the presence of serum. Medium conditioned using new P(NVP-co-EGDMA) did significantly increase the fibroblast viability in the presence of serum, whilst the P(NVP-co-DEGBAC) conditioned media had no significant effect on cell viability in the presence of serum. In the absence of serum all of the conditioned media significantly stimulated cell viability, but there was no difference between the old and new PNVP's or between the crosslinkers. These results could suggest that the polymer itself directly affects cell viability rather than the entrapment and / or release of metabolic waste or mitogens. If the polymers were trapping metabolic waste or mitogens there would be a difference in the effect of the pre-cultured polymers and the new polymers. This is not observed in either the presence or absence of serum. It is interesting to note that by day 12 it is the conditioned media that increases cell viability in the absence of serum. However in the presence of serum it is the presence of the washed polymers that increases cell viability. It is also interesting to note that the unwashed polymers had no effect on cell viability in stage 2. There was no significant effect on the water content throughout the course of the experiments indicating that over the 12 days of the experiments there was no significant bulk degradation. So whilst it appears that the polymers are directly stimulating cell viability this is not due to polymer degradation at a scale large enough to be detected via equilibrium water content measurements.

Protein adsorption studies were inconclusive as no bands could be seen in the channels into which the polymers were placed. It could therefore be suggested that either the polymers are not binding any proteins or that any proteins bound are bound so tightly that the electrical field, Tris HCl, SDS, and β–mercaptoethanol are not sufficient to separate the proteins from the polymers.

The P(NVP-co-EGDMA) hydrogel whilst non-cytotoxic does not have sufficient material properties for use as a wound dressing, however two hydrogels have now been developed that have sufficient material properties for use as a wound dressing,

P(NVP-co-DEGBAC) and P(NVP-co-DEGBAC-co-AA). These hydrogels are also non-cytotoxic when cells are grown in indirect contact with the polymers but also largely non-fouling, an ideal property in a wound dressing. These hydrogels can also stimulate cell viability and this effect is especially potent in serum free conditions.

5. The effect of β-aminopropionitrile and 4-methyl umbelliferone on cell viability, proliferation, and differentiation.

In the current experiments cell viability was assessed as before using the MTT-ESTA assay. Keratinocyte proliferation was assessed by investigating the total DNA content of cultures treated with the anti-contraction agents. Keratinocyte differentiation was assessed by investigating involucrin expression in treated cultures. Involucrin is a soluble cytoplasmic protein which is crosslinked by transglutaminase during the production of the cornified envelope formed during keratinocyte differentiation. It is expressed in a range of stratified squamous epithelia and is expressed in the suprabasal layers of stratified squamous epithelium (Griffin and Harris 1992; Carroll, Albers et al. 1993). According to Griffin et al. (1992) involucrin is normally expressed *in vivo* when cells have lost the ability to divide and is therefore considered a marker of terminal keratinocyte differentiation.

5.1 Effect of βAPN on cell viability, proliferation and differentiation.

5.1.1 Effect of βAPN on cell viability.

In these experiments the cytotoxic effects of β-aminopropionitrile (βAPN) on fibroblasts and keratinocytes was investigated using the MTT-ESTA assay. Cells were cultured in monolayer in appropriate media supplemented with βAPN at concentrations of 0-800 μg.ml^{-1}. Cells were cultured individually and in a 3:1 co-culture of keratinocytes and fibroblasts for 7 days and the results are shown in figure 5.1 below. These data show that βAPN at concentrations of between 50 and 800 μg.ml^{-1} does not have a significant effect on the viability of these cells.
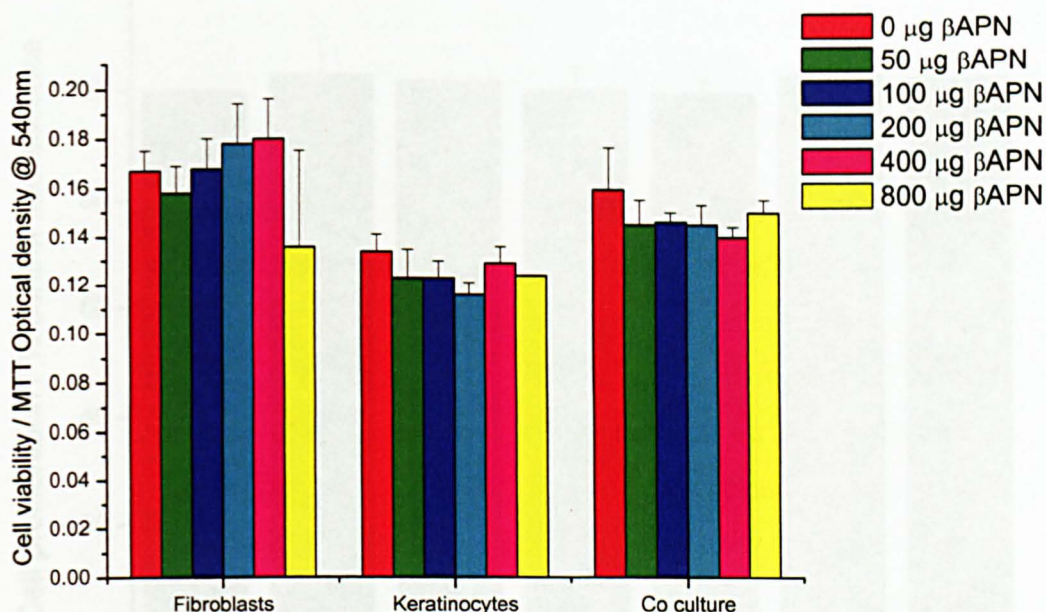

Figure 5.1 Effect of increasing concentrations of βAPN on viability of fibroblasts, keratinocytes and 3:1 co-culture of keratinocytes and fibroblasts. Viability assessed using MTT-ESTA assay. Data expressed as mean ± SEM of n=3 experiments (3 replicates per experiment). βAPN had no significant affect on cell viability at concentrations between 50 and 800 μg.ml^{-1}.

5.1.2 Effect of βAPN on keratinocyte proliferation.

In these experiments the effect of βAPN on the proliferation of keratinocytes was investigated by measuring total DNA in the cultures. Cells were cultured in monolayer in Greens medium supplemented with βAPN at concentrations of 0-800 $\mu g.ml^{-1}$ for 7 days. DNA content was measured by lysing the cells with a buffer made from SDS, Urea and PBS and then treating the lysate with DAPI. The results are shown in figure 5.2 below. DAPI is a fluorescent stain which binds strongly to DNA. These data show that βAPN at concentrations of between 25 and 800 $\mu g.ml^{-1}$ does not have a significant effect on the proliferation of keratinocytes.
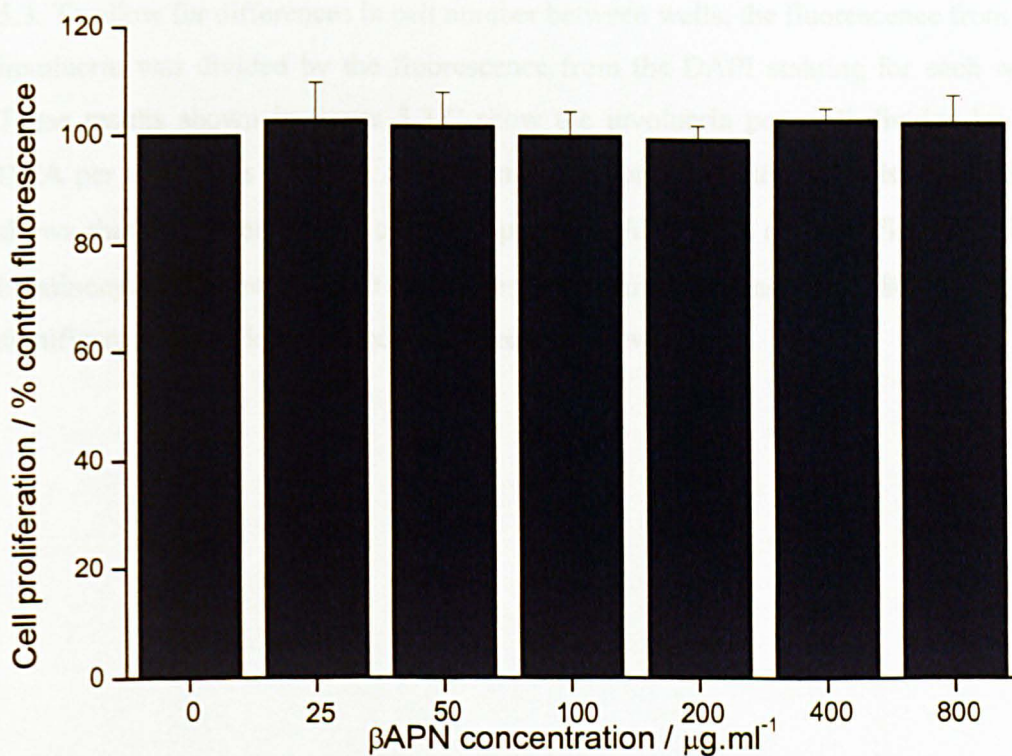

Figure 5.2 Effect of increasing concentrations of βAPN on the proliferation of keratinocytes. Proliferation was assessed by measuring total DNA. Data expressed as mean ± SEM of n=3 experiments (3 replicates per experiment). βAPN had no significant affect on cell proliferation at concentration between 25 and 800 $\mu g.ml^{-1}$ as assessed by Students' T-Test.

5.1.3 Effect of βAPN on keratinocyte differentiation.

In these experiments the effect of βAPN on the differentiation of keratinocytes was investigated by immunolabelling of the involucrin expression in the cultured cells. Cells were cultured in monolayer in Greens medium supplemented with βAPN at concentrations of 0-800 μg.ml^{-1} for 7 days. Photomicrographs were subsequently taken of the fixed cells which were co-stained with DAPI to enable identification of the cell nucleus. These are shown in figure 5.4 below. After the photomicrographs were taken the fluorescence from the cells was read in a plate reader to allow the results to be quantified. The graphs obtained from these results are shown in figure 5.3. To allow for differences in cell number between wells, the fluorescence from the involucrin was divided by the fluorescence from the DAPI staining for each well. These results shown in figure 5.3.C show the involucrin per well divided by the DNA per well gives an index of differentiation for the remaining cells. These data shows that at concentrations of 0-400 μg.ml^{-1} βAPN does not significantly affect keratinocyte differentiation. At the highest concentration investigated, 800 μg.ml^{-1}, a significant increase in keratinocyte differentiation was seen.
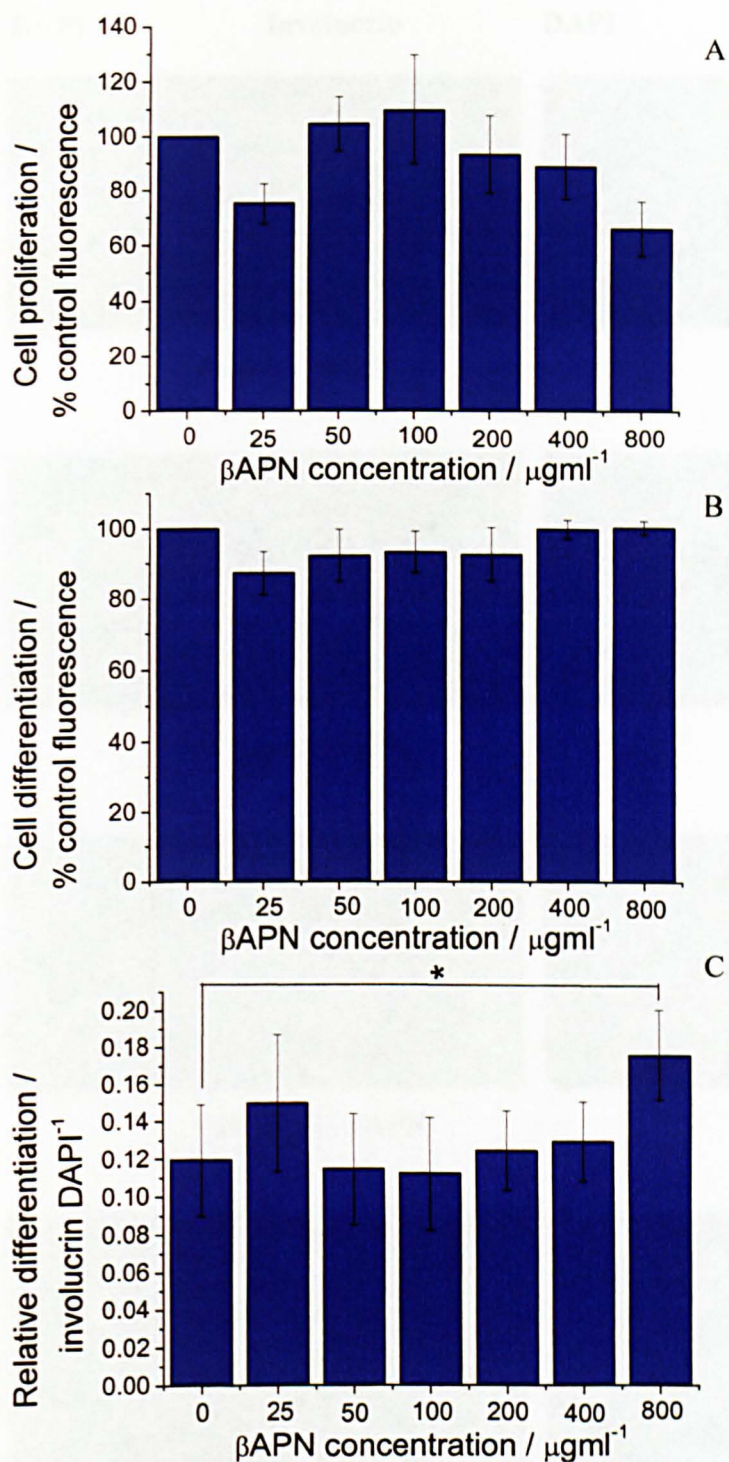
Figure 5.3 Effect of increasing concentrations of βAPN on the differentiation of keratinocytes over 7 days. Data expressed as mean ± SEM of n=3 experiments (3 replicates per experiment) A) DAPI fluorescence staining showing proliferative cells present expressed as a % of the TCP control. B) Involucrin staining showing terminally differentiating cells expressed as a % of the TCP control. C) In C the amount of involucrin per cell is estimated by showing the relative involucrin per well (expressed with respect to the TCP control) divided by the relative amount of DNA per well (expressed with respect to the TCP control).
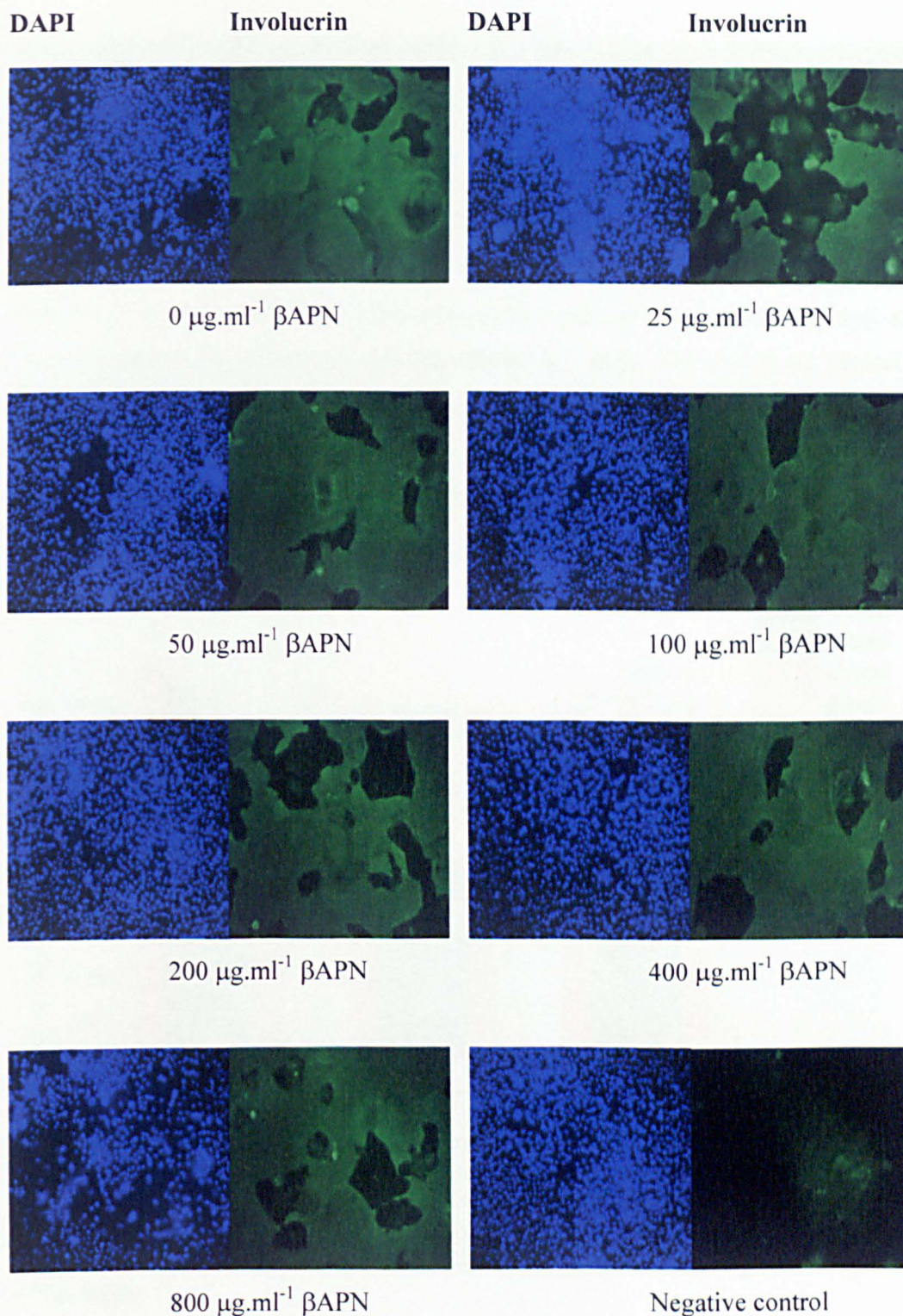
| DAPI | Involucrin | DAPI | Involucrin |

$0 \ \mu g.ml^{-1} \ \beta APN$ $25 \ \mu g.ml^{-1} \ \beta APN$

$50 \ \mu g.ml^{-1} \ \beta APN$ $100 \ \mu g.ml^{-1} \ \beta APN$

$200 \ \mu g.ml^{-1} \ \beta APN$ $400 \ \mu g.ml^{-1} \ \beta APN$

$800 \ \mu g.ml^{-1} \ \beta APN$ Negative control

Figure 5.4 Photomicrographs of keratinocytes treated with βAPN at concentrations of 0-800 $\mu g.ml^{-1}$ for 7 days. Cells were immunolabelled with DAPI to show the cell nuclei and a FITC conjugated antibody for anti-involucrin.

5.2 Effect of 4-MU on cell viability, proliferation and differentiation.

5.2.1 Effect of 4-MU on cell viability.

In these experiments the cytotoxic effects of 4-methyl umbelliferone (4-MU) on human skin cells, fibroblasts and keratinocytes was investigated using the MTT-ESTA assay. Cells were cultured in monolayer in appropriate media supplemented with 4-MU at concentrations of 0-8 mM. Cells were cultured individually and in a 3:1 co-culture of keratinocytes and fibroblasts for 7 days. The results are shown in figure 5.5 below. It can be clearly seen that at concentrations of 4-MU greater than or equal to 1 mM the cell viability was significantly reduced. Having cells in co-culture did not protect against the cytotoxic effects of the 4-MU.
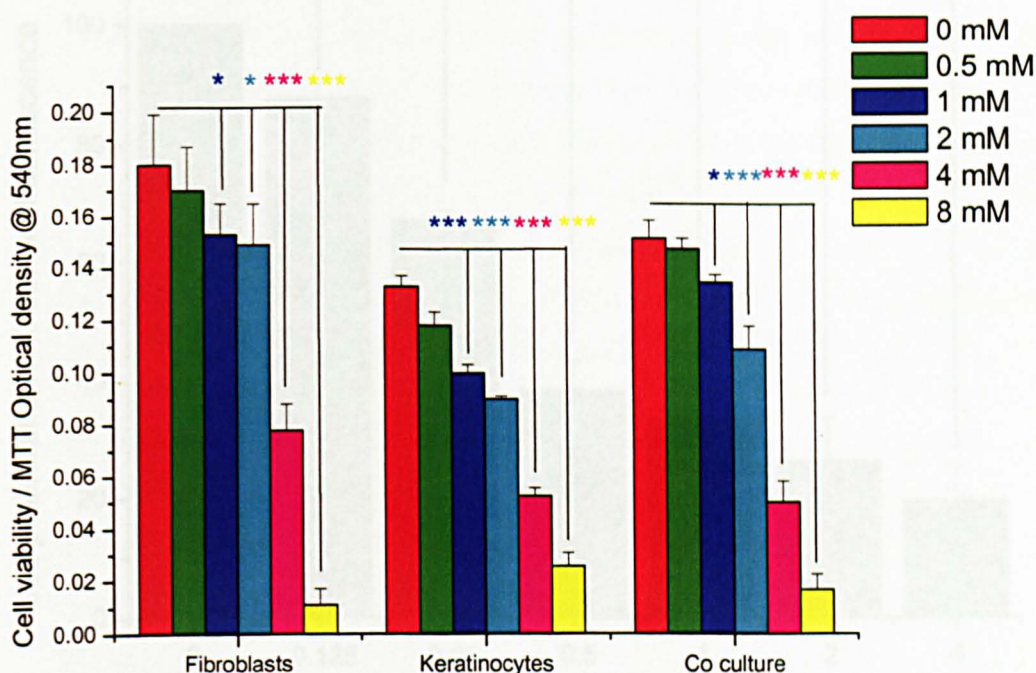

Figure 5.5 Effect of increasing concentrations of 4-MU on viability of human skin cells, fibroblasts, keratinocytes and 3:1 co-culture of keratinocytes and fibroblasts. Viability assessed using MTT-ESTA assay. Data expressed as mean ± SEM of n=3 experiments (3 replicates per experiment). * indicates significant difference in optical density from untreated culture (Student's paired T test). *p<0.05 **p<0.01 ***p<0.001.

5.2.2 Effect of 4-MU on keratinocyte proliferation.

In these experiments the effect of 4-MU on the proliferation of keratinocytes was investigated by measuring total DNA in the cultures. Cells were cultured in monolayer in Greens medium supplemented with 4-MU at concentrations of 0-4 mM for 7 days. DNA content was measured by lysing the cells with a buffer made from SDS, Urea and PBS and then treating the resulting lysate with DAPI. The results are shown in figure 5.6 below. DAPI is a fluorescent stain which binds strongly to DNA. It can clearly be seen that at concentrations of greater than 0.125 mM a significant inhibition in keratinocyte proliferation is seen.
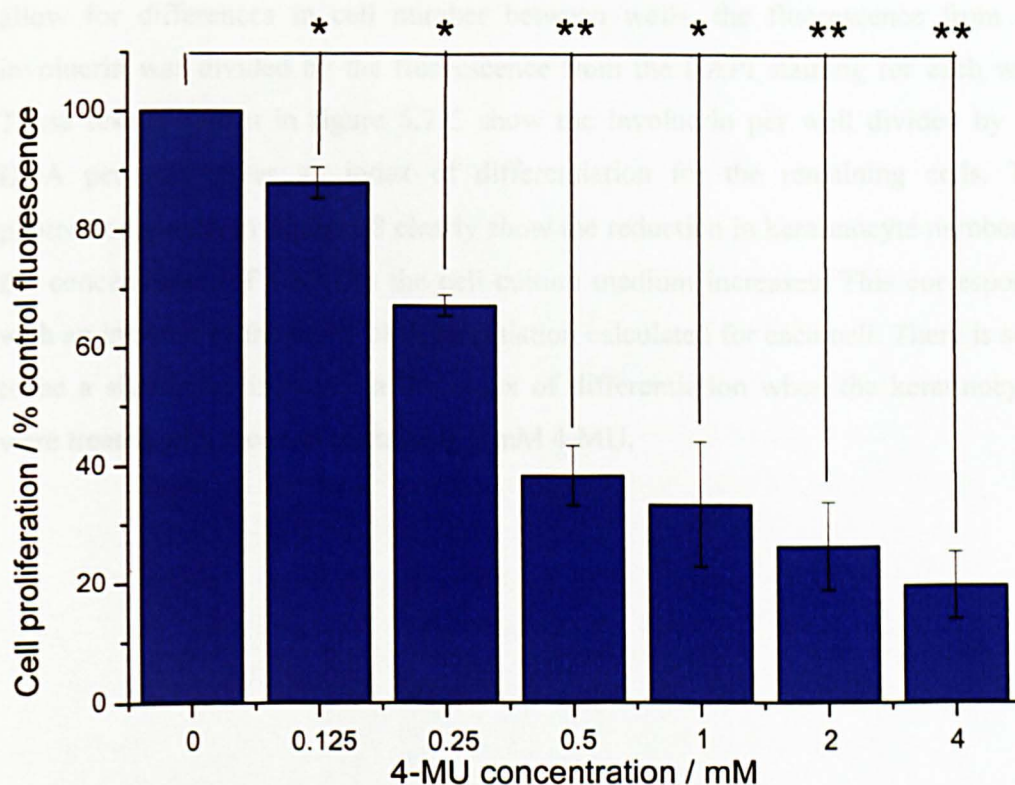

Figure 5.6 Effect of increasing concentrations of 4-MU on the proliferation of keratinocytes. Proliferation was assessed by measuring total DNA, data expressed as mean ± SEM of n=3 experiments (3 replicates per experiment). * indicates significant difference in relative fluorescence from untreated culture (Student's paired T test) *p<0.05.

5.2.3 Effect of 4-MU on keratinocyte differentiation.

In these experiments the effect of 4-MU on the differentiation of keratinocytes was investigated by immunolabelling of the involucrin expression in the cultured cells. Cells were cultured in monolayer in Greens medium supplemented with 4-MU at concentrations of 0 – 4 mM for 7 days. Photomicrographs were subsequently taken of the fixed cells which were co-stained with DAPI to enable identification of the cell nucleus. These are shown in figure 5.8 below. After the photomicrographs were taken the fluorescence from the cells was read in a plate reader to allow the results to be quantified. The graphs obtained from these results are shown in figure 5.7. To allow for differences in cell number between wells, the fluorescence from the involucrin was divided by the fluorescence from the DAPI staining for each well. These results shown in figure 5.7.C show the involucrin per well divided by the DNA per well gives an index of differentiation for the remaining cells. The photomicrographs in figure 5.8 clearly show the reduction in keratinocyte number as the concentration of 4-MU in the cell culture medium increases. This corresponds with an increase in the index of differentiation calculated for each cell. There is seen to be a significant increase in the index of differentiation when the keratinocytes were treated with medium containing 2 mM 4-MU.
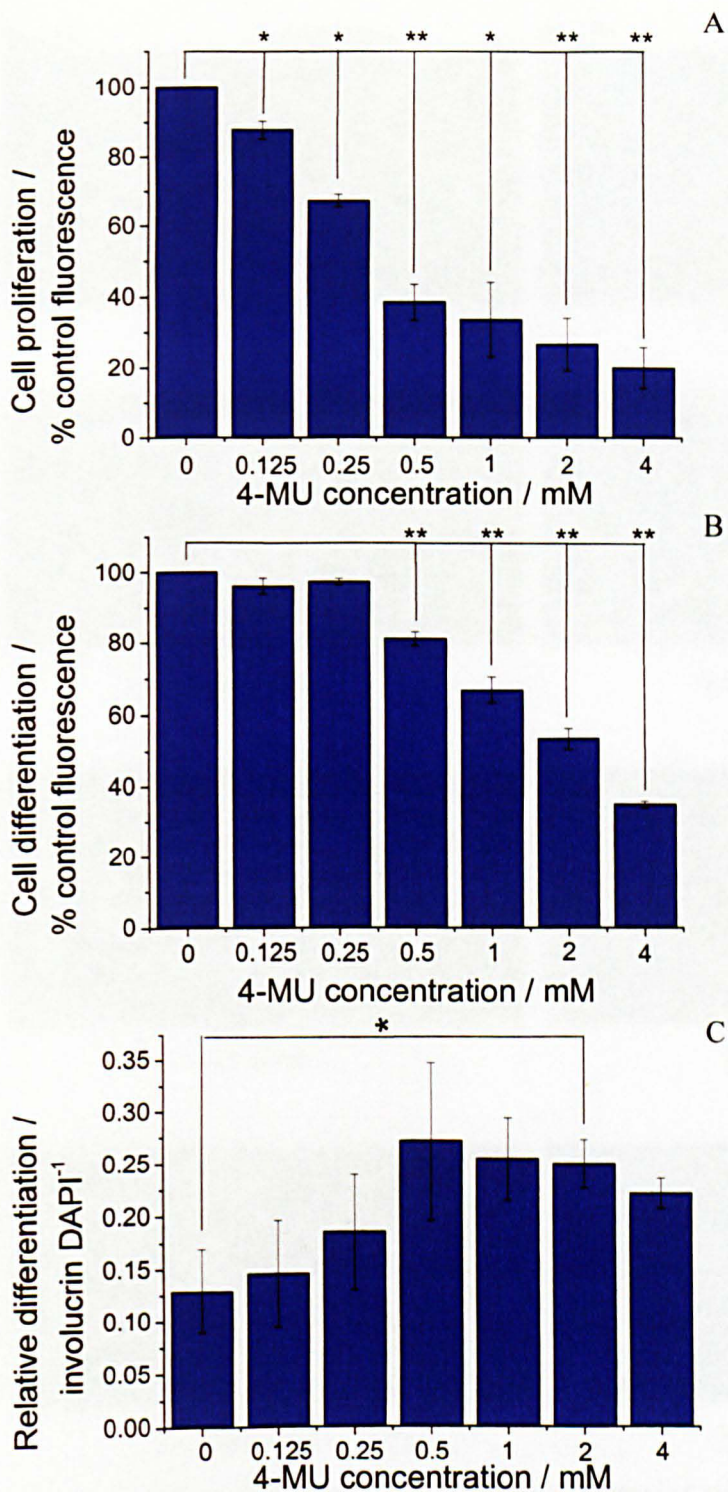
Figure 5.7 Effect of increasing concentrations of 4-MU on the differentiation of keratinocytes over 7 days. A) DAPI fluorescence staining showing proliferative cells present expressed as % TCP control. B) Involucrin staining showing terminally differentiating cells expressed as % TCP control. C) Amount of involucrin per proliferative cell. Data expressed as mean ± SEM of n=3 experiments (3 replicates per experiment). * indicates significant difference in relative fluorescence from untreated culture (Student's paired T test) *$p < 0.05$.
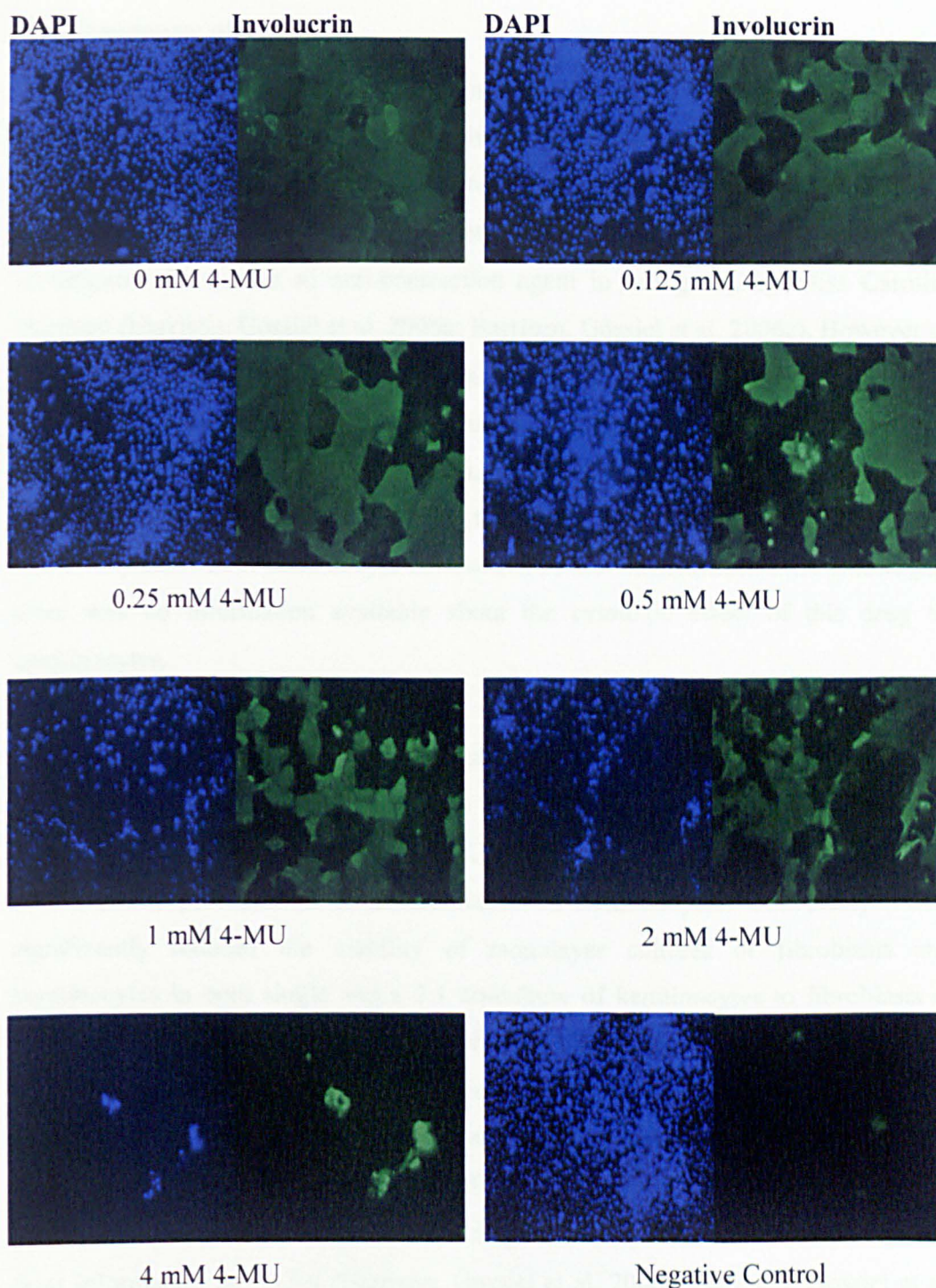
Figure 5.8 Photomicrographs of keratinocytes treated with 4-MU at concentrations of 0 – 4 mM cells were immunolabelled with DAPI to show the cell nuclei and a FITC conjugated antibody for involucrin.

5.4 Summary of results

The aim of this project is the synthesis and characterisation of a polymer membrane that could subsequently be used to deliver anti-contraction agents. Two potential anti-contraction agents were identified from previous work carried out in Professor MacNeil's laboratory and from surveying the literature βAPN had recently been investigated for use as an anti-contraction agent in this group by Miss Caroline Harrison (Harrison, Gossiel et al. 2006a; Harrison, Gossiel et al. 2006c). However up until this point no cytotoxicity testing had been undertaken with βAPN. 4-MU has been shown to reduce hyaluronan production by cells (Rilla, Pasonen-Seppanen et al. 2004; Rilla, Siiskonen et al. 2005; Nagira, Nagahata-Ishiguro et al. 2007). It was also associated with a reduction in keratinocyte differentiation and hence was postulated that it may also affect keratinocyte contraction of the reconstructed skin. Once again there was no information available about the cytotoxic effect of this drug on keratinocytes.

These experiments showed that βAPN had no significant effect on the viability of monolayer cultures of dermal fibroblasts and keratinocytes in both single and a 3:1 co-culture of keratinocytes to fibroblasts. Similarly βAPN had no significant effect on either the proliferation or differentiation of keratinocytes. Conversely 4-MU significantly reduced the viability of monolayer cultures of fibroblasts and keratinocytes in both single and a 3:1 co-culture of keratinocytes to fibroblasts at concentrations of 1 mM or greater. There was also a significant reduction in cell proliferation in monolayer keratinocyte cultures treated with 4-MU at concentration of 0.125 mM or greater. Differentiation was also reduced in these cultures however once this was corrected for the effects of 4-MU on cell number it was evident that the degree of differentiation of the remaining cells was increased. Thus in summary prior information on βAPN (Harrison, Gossiel et al. 2006a; Harrison, Gossiel et al. 2006c) showed it to have potential as an anti-contraction agent while 4-MU had not previously been tested with respect to skin contraction. However it was evident from the data from these results that in studying 4-MU the drug was clearly cytotoxic to skin cells and therefore could only be examined over a relatively narrow range of concentrations.

6. Effect of β-aminopropionitrile and 4-methyl umbelliferone on contraction.

Two contraction models were used to examine the efficacy of βAPN and 4-MU on contraction. The first model was a simple collagen gel model and the second was a more physiologically relevant reconstructed skin model.

6.1 Contraction of collagen gels.

6.1.1 Contraction of collagen gels populated with fibroblasts and keratinocytes.

As mentioned earlier the majority of research into skin contraction is performed on fibroblast populated collagen gels. It was therefore necessary to establish a more physiologically relevant collagen gel model to enable better characterisation of the drugs under consideration. To this end collagen gels were produced and seeded with human dermal fibroblasts and normal human keratinocytes in either mono or co-culture. The cells were either placed directly into the gel or on top of a precast gel. In all cases the final cell number was 5×10^4 cells. In keratinocyte containing experiments Greens medium was used to feed the cells. Fibroblast culture medium was used in fibroblast mono-culture experiments and the experiments were run for 5 days. In co culture experiments a 3:1 ratio of keratinocytes (P1 or P2) to fibroblasts (P3-9) were used. This is to mimic the seeding densities used in the production of the reconstructed skin. Image analysis was used to measure the area of the gels at 24 hour time points and the results are shown in figure 6.1.

Collagen gels without any cells present did not contract over the time period examined. Collagen gels where fibroblasts or keratinocytes were seeded on top of the gel contracted the least whilst gels populated with both cell types contracted the most. The gels where fibroblasts were seeded inside the gel contracted steadily over the culture period however when keratinocytes were seeded inside the gels a sharp

reduction in surface area was observed over the first 24 hours and very little contraction seen during the following time periods. Unfortunately the error bars for the co-culture gels are large and it is not possible to draw any firm conclusion from these experiments. However it is clear that both cell types can contract collagen I gels. When keratinocytes are placed inside the gels or cultured with fibroblasts these gels tend to contract to a greater extent than gels populated with fibroblasts alone.
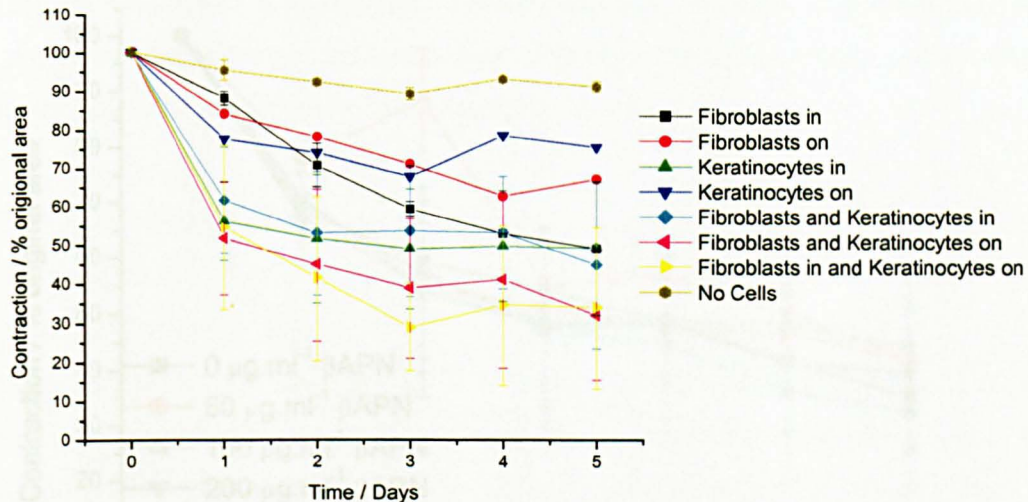

Figure 6.1 Graph showing how the cellular composition of collagen gels affects collagen gel contraction over 5 days. Data expressed as mean ± SEM of n=2 experiments (3 replicates per experiment) for all experiments except keratinocytes on and fibroblasts on (n=1). Collagen gels without any cells present did not contract over the time period examined. Collagen gels where fibroblasts or keratinocytes were seeded on top of the gel contracted the least whilst gels populated with both cell types contracted the most.

6.1.2 Effect of βAPN on collagen gel contraction.

These experiments show the effect of βAPN supplemented medium on the contraction of collagen gels populated with a 3:1 ratio of keratinocytes and fibroblasts. The medium contained βAPN at concentrations of between 50 and 800 $\mu g.ml^{-1}$. Unfortunately βAPN does not seem to affect the contraction of collagen gels populated with both cell types as shown in figure 6.2 below.
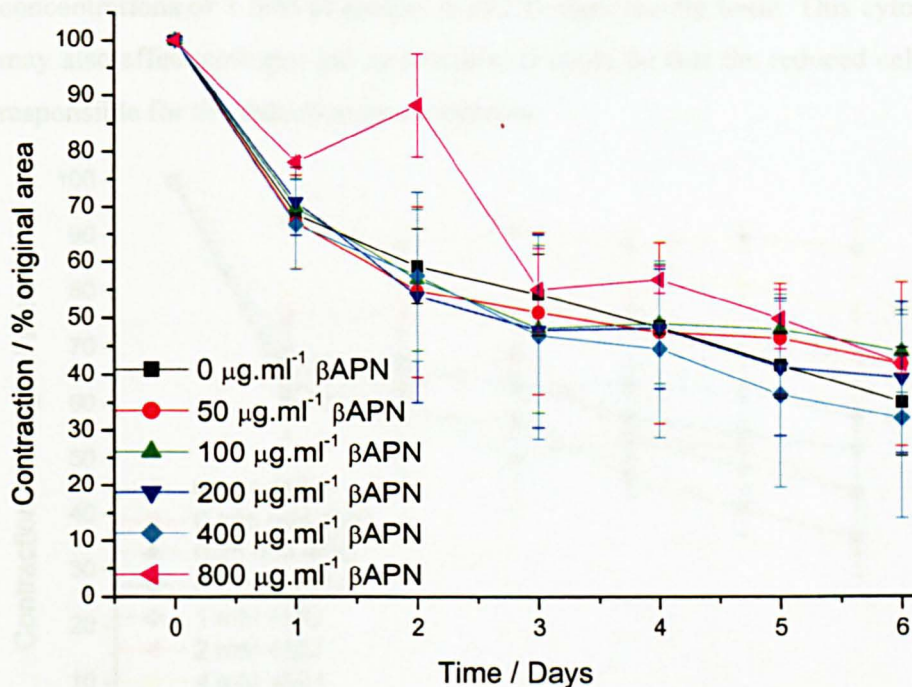

Figure 6.2 Effect of increasing concentrations of βAPN on contraction of collagen gels over a period of 6 days. Data expressed as mean ± SEM of n=3 experiments (3 replicates per experiment). On day 6 βAPN had no significant affect on the contraction of collagen gels at concentrations between 50 and 800 $\mu g.ml^{-1}$ as assessed using 1-way ANOVA + Bonferroni correction.

6.1.3 Effect of 4-MU on contraction of collagen gels

Figure 6.3 shows the effect of 4-MU supplemented medium on the contraction of collagen gels populated with a 3:1 ratio of keratinocytes and fibroblasts. The medium contained 4-MU at concentrations of between 0.125 and 4 mM. 4-MU at concentration of 1 mM and greater significantly reduced the contraction of the collagen gels. However figure 5.5 illustrates that in monolayer co-cultures at concentrations of 1 mM or greater 4-MU is significantly toxic. This cytotoxic effect may also affect collagen gel contraction. It could be that the reduced cell number is responsible for the reduction in contraction.
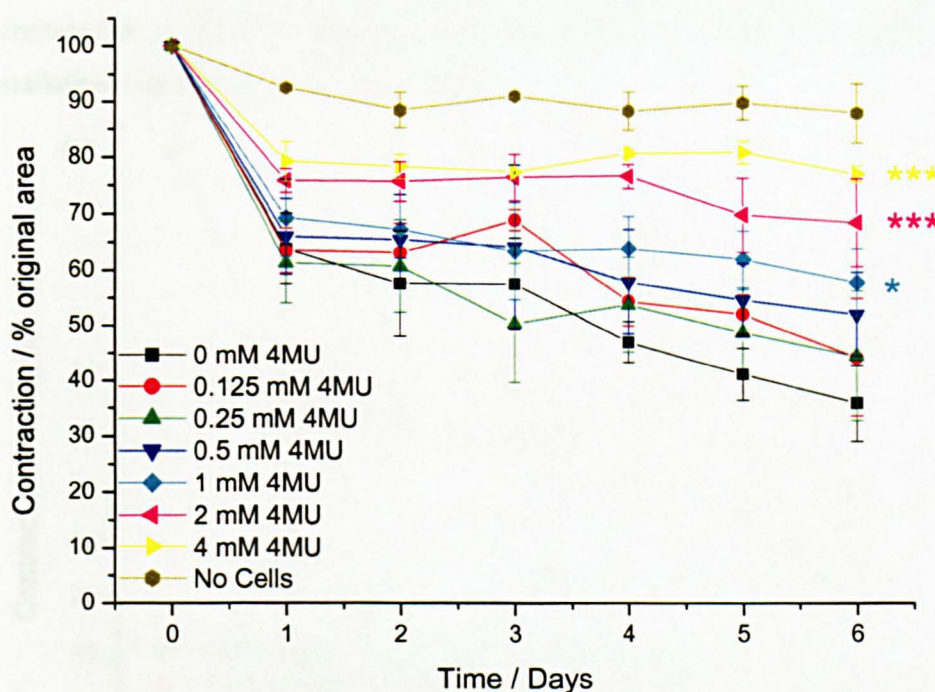

Figure 6.3 Effect of increasing concentrations of 4-MU on contraction of collagen gels over a period of 6 days. Data expressed as mean ± SEM of n=3 experiments (3 replicates per experiment). On day 6 4-MU has no significant affect on the contraction of collagen gels at concentrations between 0.125 and 0.5 mM. A significant difference in contraction at day 6 was observed in collagen gels treated with 4-MU at concentrations of 1 mM and greater. * indicates significant difference in area from untreated collagen gels (1-way ANOVA + Bonferroni correction.). *p<0.05 **p<0.01 ***p<0.001.

6.1.4 Effect of 4-MU on contraction of fibroblast populated collagen gels.

Work in the laboratory of Dr. Mike Edwards at Glasgow (data not shown and unpublished at this time) suggests medium supplemented with 2mM 4-MU would reduce the contraction of fibroblast populated collagen gels. Collagen gels were prepared containing 5×10^4 fibroblasts and cultured in 4-MU supplemented medium for 6 days. Every 24 hours the medium was completely replaced and the gels photographed for later image analysis. The data is shown in figure 6.4 below. Unfortunately, whilst there was a trend to suggest that 4-MU could reduce the contraction of fibroblast populated collagen gels the variation in the data meant that statistical significance was not obtained.
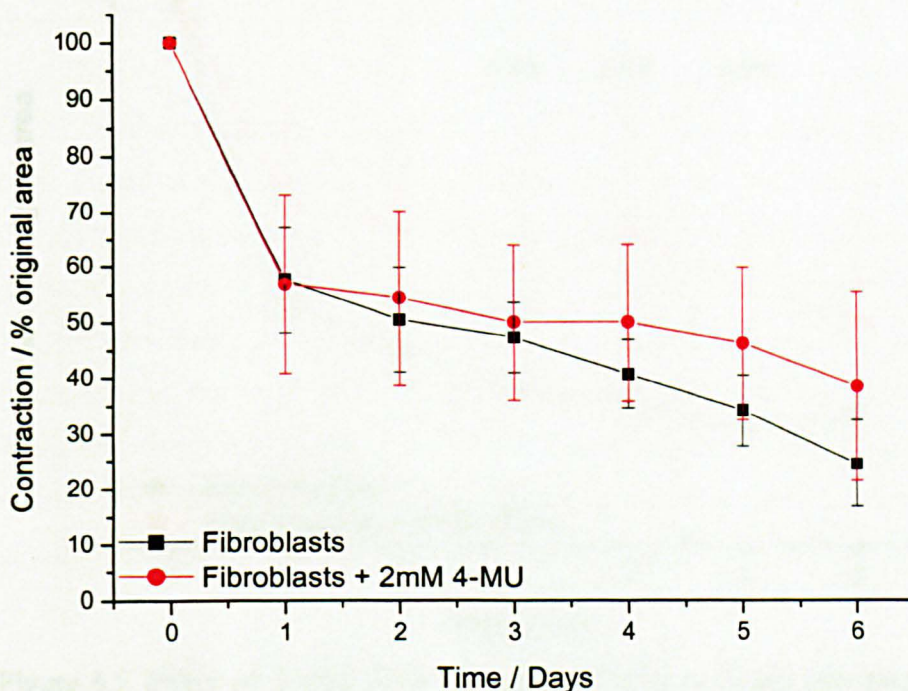

Figure 6.4 Effect of 2 mM 4-MU on contraction of collagen gels populated with fibroblasts over a period of 6 days. Data expressed as mean ± SEM of n=3 experiments (3 replicates per experiment). 4-MU has no significant effect on the contraction of collagen gels populated with human dermal fibroblasts as assessed using 1-way ANOVA + Bonferroni correction at day 6.

6.1.5 Effect of 4-MU on contraction of keratinocyte populated collagen gels.

5×10^4 keratinocytes were seeded on top of pre-cast collagen gels and cultured in *Greens* medium supplemented with 2 mM 4-MU for 6 days. Every 24 hours the medium was completely replaced and the gels photographed for later image analysis. The data is shown in figure 6.5 below. Unfortunately, again whilst there was a trend to suggest that 2 mM 4-MU could reduce the contraction of keratinocyte populated collagen gels the variation in the data meant that statistical significance was not obtained at day 6. A significant difference in contraction was observed despite the large errors between days 3 and 5. Suggesting that 4-MU at a concentration of 2 mM does significantly reduce the contraction of keratinocyte populated collagen gels.
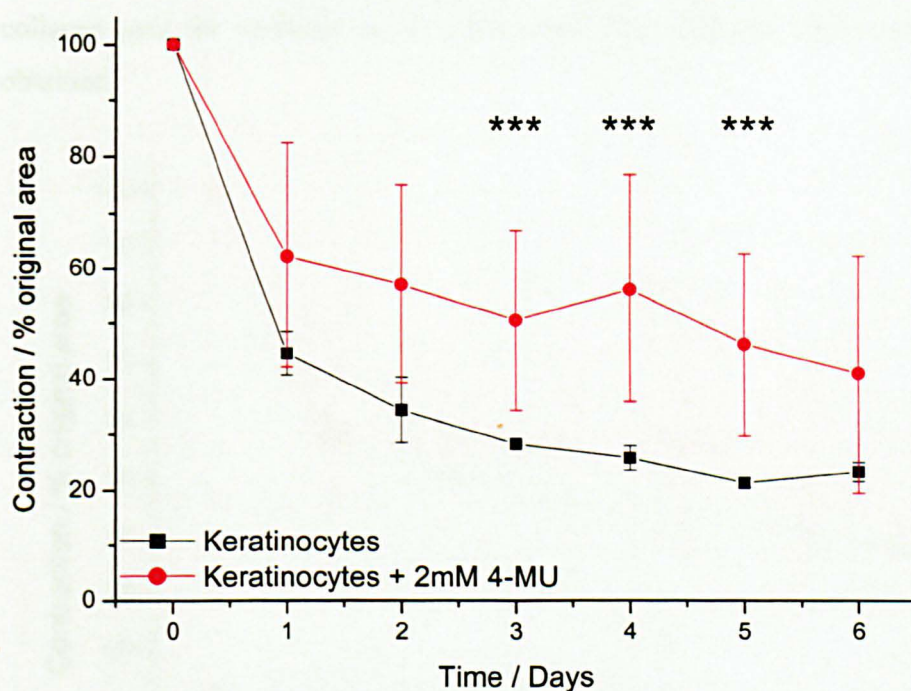

Figure 6.5 Effect of 2 mM 4-MU on contraction of collagen gels populated with keratinocytes over a period of 6 days. Data expressed as mean ± SEM of n=3 experiments (3 replicates per experiment). 4-MU has no significant effect on the contraction of collagen gels populated with keratinocytes as assessed using 1-way ANOVA + Bonferroni correction at day 6. However a significant difference in contraction was observed between days 3 and 5.

6.1.4 Effect of 4-MU on contraction of collagen gels populated with fibroblasts and keratinocytes.

Effect of 4-MU at a concentration of 2 mM on contraction of collagen gels populated with both fibroblasts and keratinocytes was also investigated. 5×10^4 cells were seeded in a 3:1 ratio of keratinocytes to fibroblasts. The fibroblasts were seeded inside the gel and the keratinocytes on top. These gels were cultured Greens medium supplemented with 2 mM 4-MU for 6 days. Every 24 hours the medium was completely replaced and the gels photographed for later image analysis. The data is shown in figure 6.6 below. Unfortunately, again whilst there was a trend to suggest that treatment of the collagen gels with 2 mM 4-MU could reduce the contraction of collagen gels the variation in the data meant that statistical significance was not obtained.
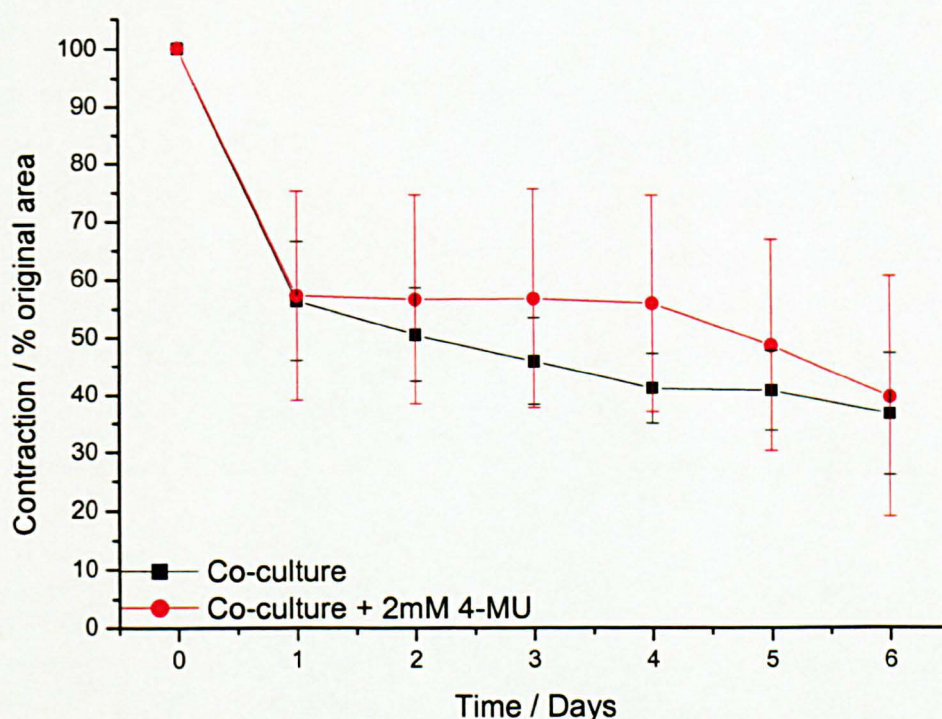

Figure 6.6 Effect of 2 mM 4-MU on contraction of collagen gels populated with a 3:1 ratio of keratinocytes: fibroblasts over a period of 6 days Data expressed as mean ± SEM of n=3 experiments (3 replicates per experiment). 4-MU has no significant effect on the contraction of collagen gels populated with keratinocytes as assessed using 1-way ANOVA + Bonferroni correction at day 6.

6.2 Reconstructed skin model

The reconstructed skin model has been established for many years in Prof. MacNeil's group. Although it has many uses, in this project it was used to model contraction. It has been clearly shown by Harrison (Harrison, Gossiel et al. 2006c), Thornton (Thornton, Harrison et al. Provisionally accepted) and Chakrabarty (Chakrabarty, Heaton et al. 2001) how this model can be used to see how various potential anti-contraction agents affect contraction. Statistics performed on these data were a 2-way ANOVA + Bonferroni correction. These statistics were chosen to allow comparison between these data and the data of Harrison et al. (Harrison, Gossiel et al. 2006c).

6.2.1 The effect of βAPN on contraction of reconstructed skin.

These experiments (carried out by Miss Caroline Harrison) show the effect of βAPN supplemented medium on the contraction of collagen gels after 28 days culture at an air-liquid interface in Greens medium. The culture medium contained βAPN at concentration of 50, 100 and 200 μg.ml^{-1}. Whilst βAPN had no significant effect on the contraction of collagen gels (see figure 6.2) Greens medium supplemented with 200 μg.ml^{-1} βAPN significantly reduced the contraction of the reconstructed skin composite shown below in figure 6.7.
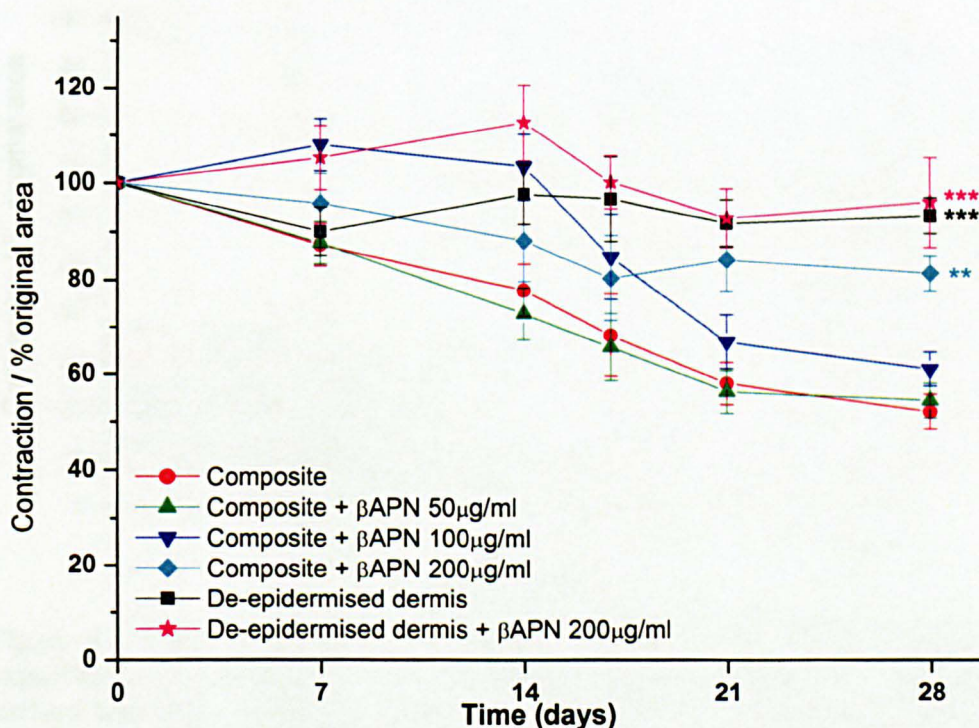

Figure 6.7 Effect of βAPN on contraction of the reconstructed skin model n=3 experiments (3 replicates per experiment). * indicates significant difference in surface area from untreated composite (2-way ANOVA + Bonferroni correction). *p<0.05 **p<0.01 ***p<0.001. No significant difference between βAPN treated and untreated DED (p>0.05). Data courtesy of Miss Caroline Harrison (Harrison, Gossiel et al. 2006c). βAPN supplemented medium (200 μg.ml^{-1}) significantly reduced the contraction of the reconstructed skin composite.

6.2.1 The effect of 4-MU on contraction of reconstructed skin.

These experiments show the effect of 4-MU supplemented medium on the contraction of the reconstructed skin model after 28 days culture at an air-liquid culture in Greens medium. The medium contained 4-MU at concentration of 0.5, 1 and 2 mM. The data is shown in figure 6.8 below. Greens medium supplemented with 4-MU at concentration of 1 mM or greater significantly reduced the contraction of the reconstructed skin composite.

Figure 6.8 Effect of 4-MU on contraction of the reconstructed skin model n=3 experiments (3 replicates per experiment). * indicates significant difference in surface area from untreated composite (1-way ANOVA + Bonferroni correction). *p<0.05 **p<0.01 ***p<0.001. There was no significant difference between 4-MU treated and untreated DED (p>0.05). Medium supplemented with 4-MU at concentrations of 1 mM and greater significantly reduced the contraction of the reconstructed skin.

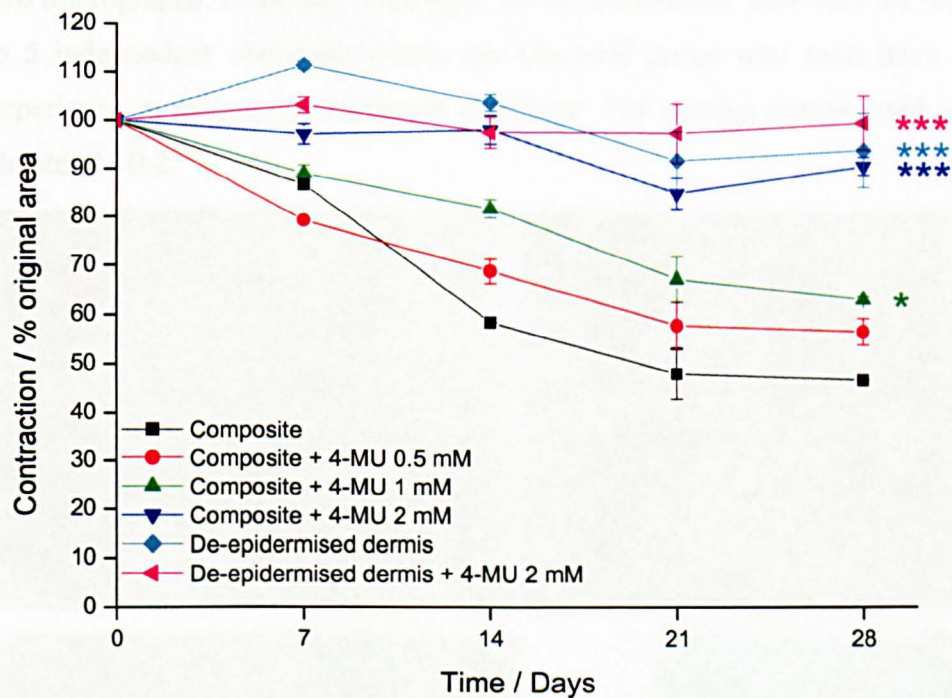## 6.2.2 Effect of 4-MU on histology of reconstructed skin.

The effects of medium supplemented with 4-MU at concentrations of 0, 0.5, 1 and 2 mM are shown in figure 6.9 below. Composites were cultured for 28 days at an air-liquid interface in Greens medium. Samples were subsequently fixed, embedded, mounted, and stained with Haematoxylin and Eosin (H&E). These sections were then photographed and sent for blind scoring. The results of the scoring are shown in the graphs in figure 6.10. To enable fair scoring with the small number of samples two micrographs, randomly numbered, for each condition were sent for blind scoring to 5 independent observers within the MacNeil group who each have significant experience in examining composite histology. The scoring system used is shown in chapter 2.10.2.
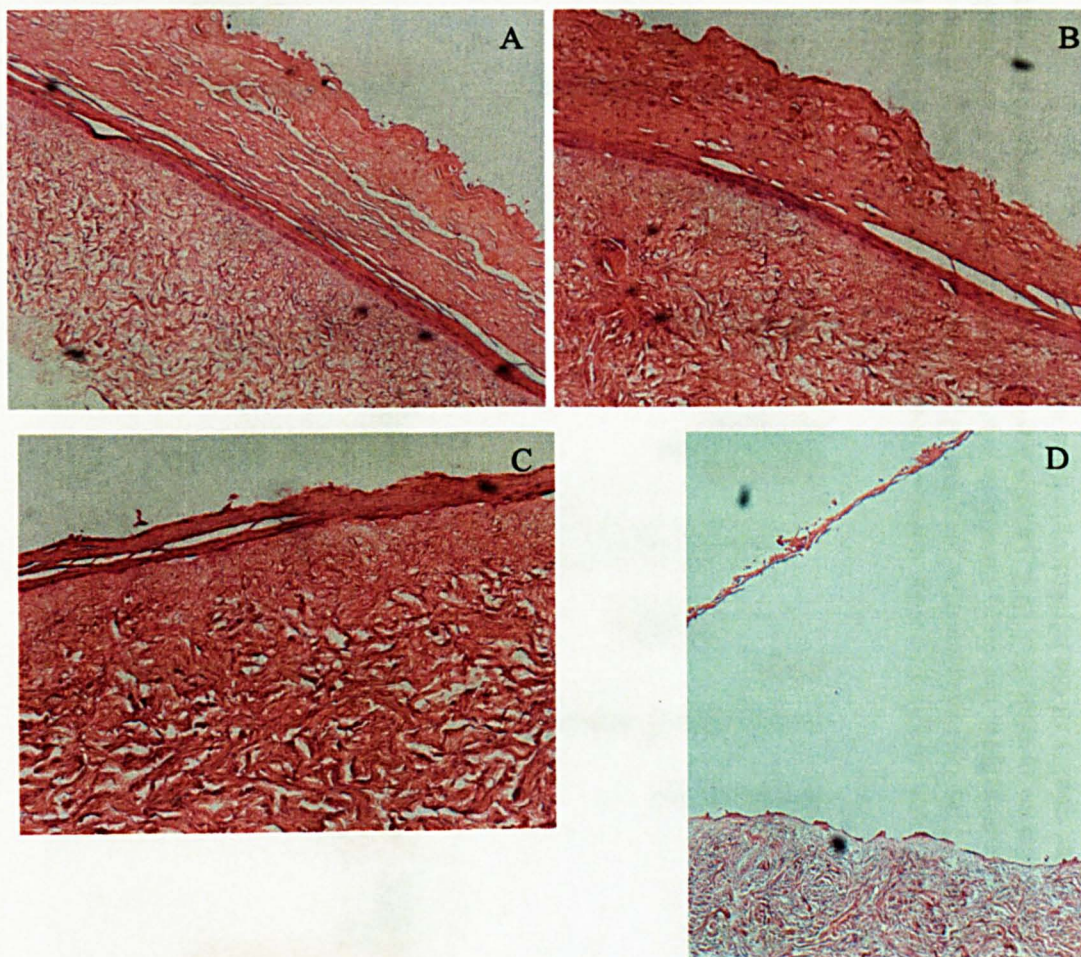

Figure 6.9 Effect of 4-MU on histology of the reconstructed skin model after 28 days culture at air-liquid interface. A) 0 mM 4MU, B) 0.5 mM 4-MU, C) 1 mM 4-MU D) 2 mM 4-MU.
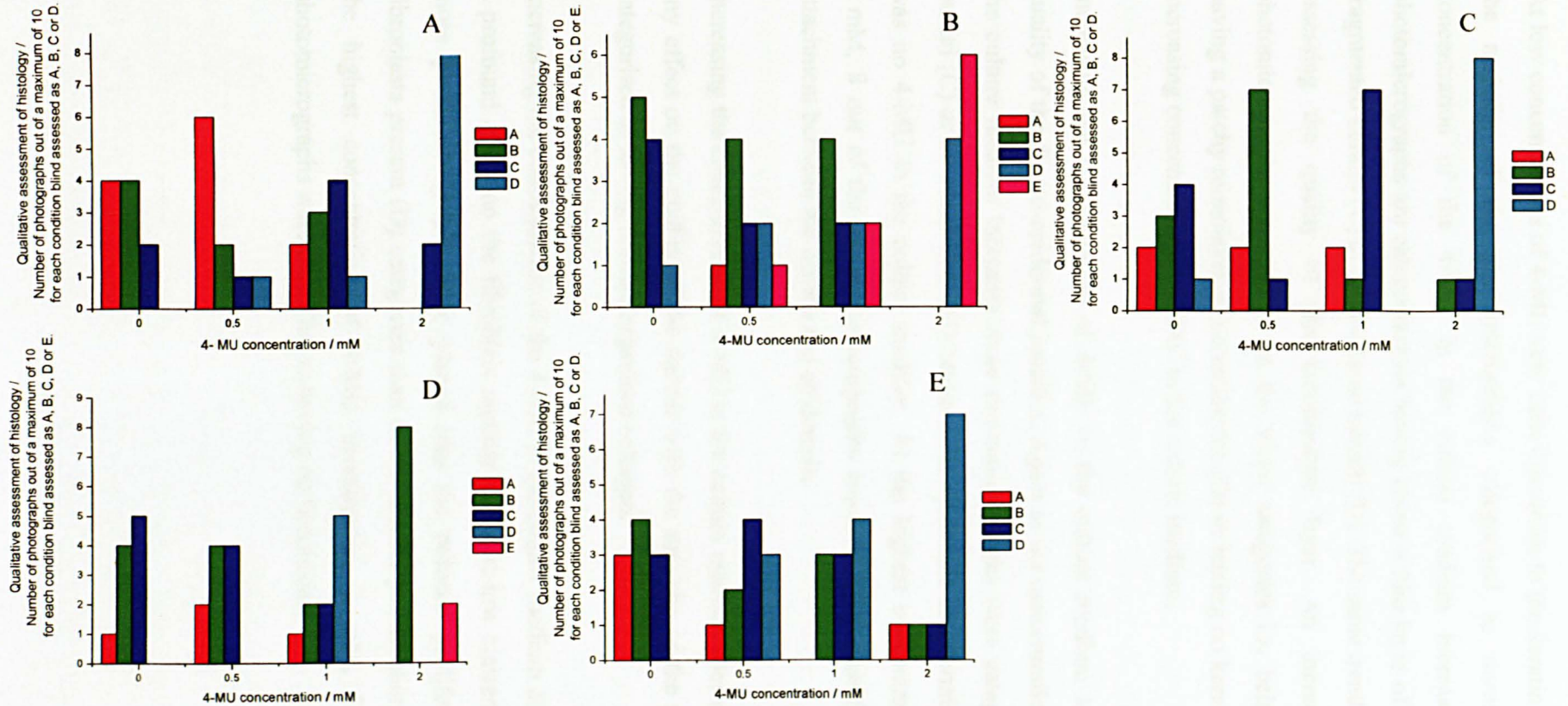
Figure 6.10 Graphs showing the qualitative assessment of histology. A) Assessment of keratin layer, B) assessment of the keratinocyte layers, C) assessment of the quality of the dermo-epidermal junction, D) assessment of the quality of the dermis, E) assessment of fibroblast number in the composites. Two photomicrographs for each condition were assessed blind by 5 independent members of the MacNeil group and scored according to the system shown in section 2.10.2. Graphs have been drawn to illustrate the number of times a photograph was categorised as A, B, C, D or in the case of the quality of the keratinocyte layer and the dermis E.

At low concentrations of 4-MU very little disruption to the keratin layer is seen with the majority of the photomicrographs categorised as normal (A). As the concentration of the 4-MU in the culture medium increases more of the photomicrographs are categorised as having either a thin layer of poorly adherent / fragmented keratin (C) or as having no keratin (D). The same trend is observed when assessing the quality of the keratinocyte layer. An increased number of photomicrographs were placed in the worst categories i.e. being categorised as having a patchy monolayer of keratinocytes (D) or having no keratinocytes (E) with increasing concentrations of 4-MU in the culture medium.

Increasing the concentration of 4-MU in the culture medium also decreases the quality of the dermo-epidermal junction. Again as the concentration of the 4-MU in the culture medium increases more photomicrographs were categorised as having partial (C) or no attachment (D) between the epidermis and dermis than when there was no 4-MU in the culture medium. At the highest concentration investigated, 2 mM, 8 out of the 10 photomicrographs assessed were classified as having no attachment between the dermis and epidermis.

Increasing the concentration of 4-MU in the culture medium does not appear to have any effect on the quality of the dermis with the majority of the photomicrographs categorised as having normal, organised collagen.

Increasing the concentration of the 4-MU in the culture medium does appear to have a profound effect on the fibroblast number. Even at low concentrations of 4-MU more photomicrographs were placed into the reduced proliferation (C) or no fibroblasts present (D) categories than in the normal proliferation (B) category. At the highest concentration of 4-MU investigated, 2 mM, 7 out of the 10 photomicrographs were classified as having no fibroblasts.

6.3 Summary of Results.

Initially the collagen gel model was used to examine the efficacy of the two anti-contraction agents under investigation, β-APN and 4-MU. The fibroblast populated collagen gel model is a cheap, simple model commonly used to study skin graft contraction. However, it does have its limitations; these include the presence of only one cell type, the lack of dermal architecture and basement membrane. The lack of dermal architecture and basement membrane cannot be easily rectified. A second cell type, the keratinocyte, can relatively easily be introduced into the collagen gels. It was therefore necessary to determine how the addition of keratinocytes into fibroblast populated collagen gels affected contraction. Collagen gels were produced with fibroblasts and keratinocytes in single and co-culture. All of the collagen gels produced had an initial seeding density of 5×10^4 cells. Whilst there was no significant difference observed due to low replicate numbers and large errors the collagen gels seeded with both cell types tended to contract more than those seeded with a single cell type. The collagen gel model with fibroblasts seeded in the gel and keratinocytes seeded on top of the gel in a 3:1 ratio (keratinocytes : fibroblasts) was therefore used to evaluate the efficacy of the two anti-contraction agents.

βAPN had no effect on the contraction of collagen gels populated with both fibroblasts and keratinocytes. The 4-MU significantly reduced collagen gel contraction at concentrations of 1 mM or greater. Collagen gels populated with single or co-cultures of fibroblasts and / or keratinocytes were also treated with 4-MU at a concentration of 2 mM. Unfortunately due to variation in the 4-MU treated collagen gels no statistical significance was obtained at day 6. However, in the keratinocyte populated gels a significant reduction in contraction was seen between days 3 and 5 despite the considerable variation present in the data. 4-MU also significantly reduced the contraction of the reconstructed skin model at concentrations of 1 mM or greater. Harrison et al. (Harrison, Gossiel et al. 2006c) had previously shown that βAPN at concentrations of 200 $\mu g.ml^{-1}$ can significantly reduce contraction in the same reconstructed skin model. Table 6.1 summarises the effects of βAPN and 4-MU at various concentrations on the contraction of collagen

gels populated with single and co-cultures of fibroblasts and keratinocytes, and the reconstructed skin model.

Table 6.1 Effect of βAPN and 4-MU at various concentrations on the contraction of the reconstructed skin model and collagen gels populated with single and co-cultures of fibroblasts and keratinocytes.

Drug	Model used	Cells used,	Effect on contraction
βAPN 50-800 µg.ml^{-1}	Collagen I gels	3:1 co-culture	No significant effect
4-MU 0.125-4 mM	Collagen I gels	3:1 co-culture	1 mM and 4 mM significantly reduced contraction
4-MU 2 mM	Collagen I gels	Fibroblasts	No significant effect
4-MU 2 mM	Collagen I gels	Keratinocytes	No significant effect
4-MU 2 mM	Collagen I gels	3:1 co-culture	No significant effect
βAPN* 50-200 µg.ml^{-1}	Reconstructed skin	Fibroblasts & keratinocytes	200 µg.ml^{-1} βAPN significantly reduced contraction
4-MU 0.5-2 mM	Reconstructed skin	Fibroblasts & keratinocytes	1 mM and 2 mM significantly reduced contraction

* Data courtesy of Miss Caroline Harrison (Harrison, Gossiel et al. 2006c).

Whilst the 4-MU significantly reduced the contraction of the reconstructed skin model it is important to make sure that there is no significant change to the histology of the reconstructed skin. H&E were used to stain the sections cut from the reconstructed skin composites after 28 days culture at an air-liquid interface. Photomicrographs of these sections were sent for independent blind scoring to 5 members of the MacNeil group who each have significant experience examining composite histology. The photomicrographs appear to show a dramatic change in the quality of the epidermis, both in the quality of the keratinised layer and in the keratinocytes, as the concentration of 4-MU in the medium increases. Again the quality of the dermo-epidermal junction decreases with increasing concentration of 4-MU. No difference was observed in the quality of the dermis at any of the

examined concentrations of 4-MU. However, the fibroblast number reduced dramatically when the composites were treated with 4-MU at concentrations of 1 mM or greater. Harrison et al. (2006c) also showed that treatment of the reconstructed skin with 200 μg.ml^{-1} βAPN had no significant effect on the composite morphology.

7. Release of β-aminopropionitrile and 4-methyl umbelliferone from poly(vinylpyrrolidinone) hydrogels and their effect on collagen gel contraction.

The aim of this project was the development of a hydrogel wound dressing that could potentially be used to prevent / reduce skin graft contracture. A noncytotoxic poly (vinylpyrrolidinone) (PNVP) hydrogel has been developed with suitable material properties for use as a wound dressing. Two potential anti contraction agents have been identified, β-aminopropionitrile (βAPN) and 4-methyl umbelliferone (4-MU). βAPN has been shown to be effective in reducing contraction of the reconstructed skin model (See chapter 6.2.1) but was unsuccessful in reducing the contraction of the collagen gel model (See chapter 6.1.2). 4-MU reduced contraction in both the collagen gel and reconstructed skin models (See chapter 6).

Initial experiments to quantify βAPN release into aqueous media for this project involved attempted extraction of the βAPN from the aqueous media, (cell culture medium and PBS) into organic solvent, chloroform, for gas chromatography. Unfortunately the extraction proved unsuccessful, the βAPN was still present in the aqueous media even after treatment of the aqueous medium with potassium carbonate in an attempt to free the βAPN from the fumarate. The presence of βAPN in the aqueous solution was confirmed by the reaction of the amine group with 2,4,6-trinitrobenzenesulfonic acid (TNBS). This yellow solution reacts with the amine to form an orange derivative as follows.
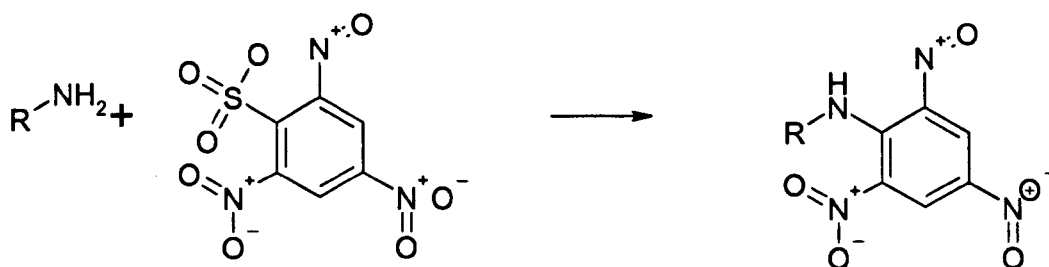

Figure 7.1 Reaction scheme for the reaction of 2,4,6-trinitrobenzenesulfonic acid with a primary amine (Hermanson 1996).

The TNBS assay has been used for many years to measure the free amino groups in proteins (Habeeb 1966). It can also be used as a qualitative check for the presence of amines, sulfhydryls, or hydrazides (Hermanson 1996). Further studies showed that this TNBS assay could be used to monitor the release of the βAPN into amine free aqueous media.

In monitoring the release of 4-MU into various aqueous media advantage was taken of the fluorescent properties of the 4-MU. Friedberg and Shihabi (1997) use 4-MU conjugated to glucosaminide as a synthetic substrate to monitor the activity of N-acetyl-β-glucosaminidase. The product of this reaction is 4-MU and UV absorption or fluorescence spectroscopy techniques can be used to quantify the amount of 4-MU in solution. This assay has also been used by Calvitti et al. (2004). Lovdahl et al. (1994) used a HPLC method to quantify the amount of 4-MU in Williams E Media and dog plasma. However, this method was not explored in this study due to potential difficulties in the extraction of the 4-MU into acetonitrile.

7.1 Drug release assay development.

7.1.1 Assay development for monitoring βAPN release.

The TNBS assay is used to determine the presence of free amino groups in amino acids and peptides. Initial experiments were performed with a protein control, BSA, to ensure that the assay worked. Initial experiments as illustrated in figure 7.2.A showed that the assay was more sensitive to βAPN than to BSA. It is thought that this is due to the protonated nitrogen in the βAPN causing the βAPN to be a more reactive species than the primary amines the assay was designed to measure. Further experiments were conducted to establish by what factor the experimental samples would need to be diluted by to prevent the saturation seen in figure 7.2.A. Dilutions of both the βAPN solutions in PBS and the TNBS solution were tried. This showed that dilution of the TNBS solution did not affect the concentration at which saturation occurred it only lowered the sensitivity of the assay. Dilution of the βAPN samples from a known concentration by 1:10 did produce a curve suitable for use.

This means that the maximum detectable βAPN concentration for these experiments is 120 μg.ml^{-1} as shown in figure 7.2.B.

A variety of other aqueous media were also tried to see which would be the best for a) releasing the βAPN into and b) diluting the samples by. Media tried were distilled water, PBS, PBS + 10% FCS, serum free DMEM, fibroblast culture medium, and Greens medium. The serum free DMEM, fibroblast culture medium, and Greens medium were investigated both with and without phenol red. In all cases except water and PBS the assay did not detect the presence of βAPN. This was thought to be due to the presence of the amino acids in these media. The phenol red also quenched the majority of the signal from the TNBS (data not shown). It was found that the best medium for the βAPN release assay was therefore PBS due to the similarities between this medium and the cell culture medium. The best medium for the sample dilution was the carbonate buffer used to make the TNBS solution used in the assay (data not shown). In all cases the experiments were performed using a 96 well plate, absorption was measured using a U.V. plate reader at 320 nm.
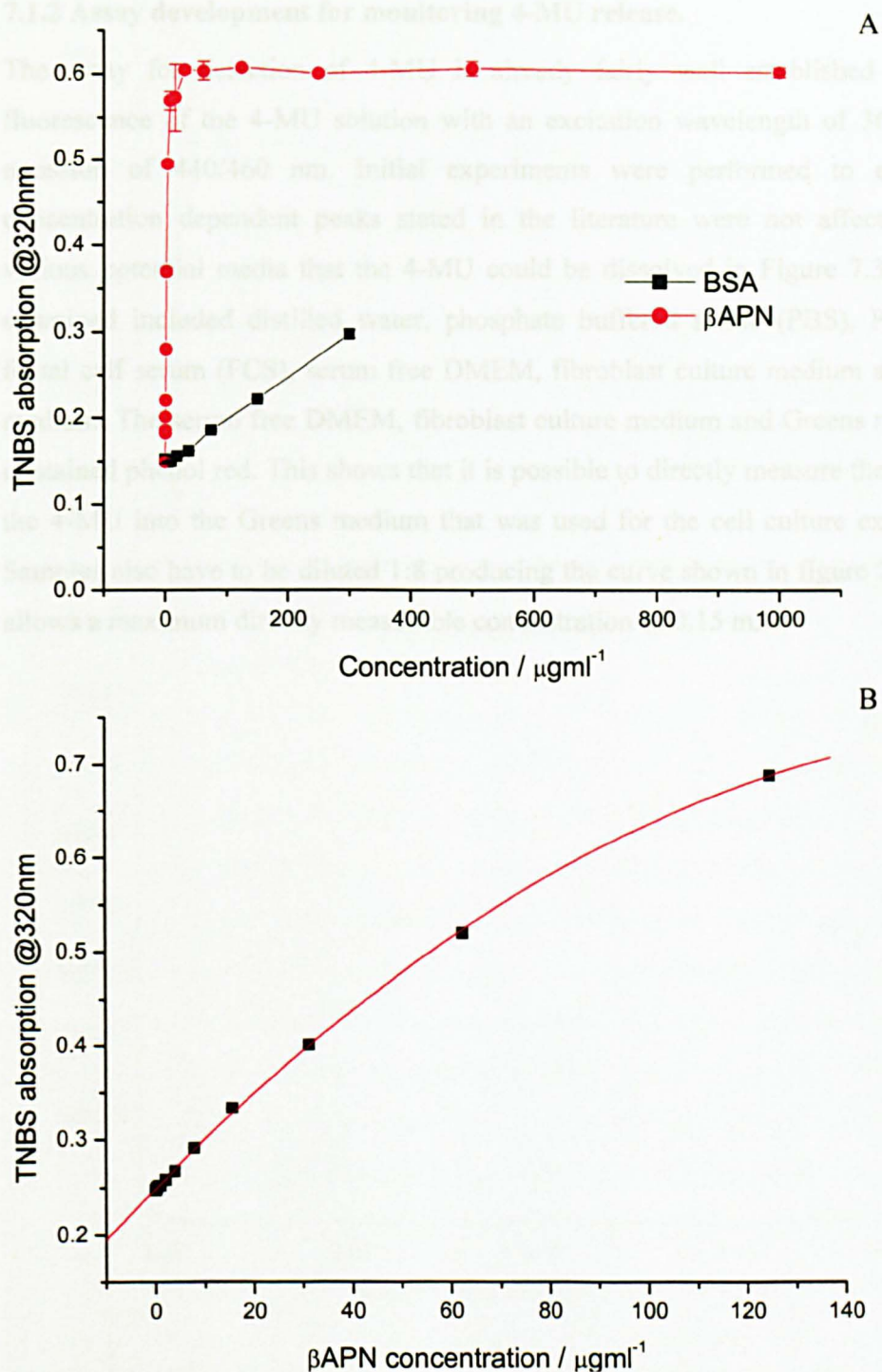
Figure 7.2 Development of the calibration curve for detection of βAPN in phosphate buffered saline (PBS) by TNBS. A) Detection of βAPN and BSA (n=6), B) Final βAPN calibration curve, samples diluted down to final concentrations using Na(CO-3)2 buffer pH 8.5 (n=6). All data expressed as mean ± SEM.

7.1.2 Assay development for monitoring 4-MU release.

The assay for detection of 4-MU is already fairly well established using the fluorescence of the 4-MU solution with an excitation wavelength of 360 nm and emission of 440/460 nm. Initial experiments were performed to ensure the concentration dependent peaks stated in the literature were not affected by the various potential media that the 4-MU could be dissolved in Figure 7.3.A. Media examined included distilled water, phosphate buffered saline (PBS), PBS +10% foetal calf serum (FCS), serum free DMEM, fibroblast culture medium and Greens medium. The serum free DMEM, fibroblast culture medium and Greens medium all contained phenol red. This shows that it is possible to directly measure the release of the 4-MU into the Greens medium that was used for the cell culture experiments. Samples also have to be diluted 1:8 producing the curve shown in figure 7.3.B. This allows a maximum directly measurable concentration of 0.15 mM.
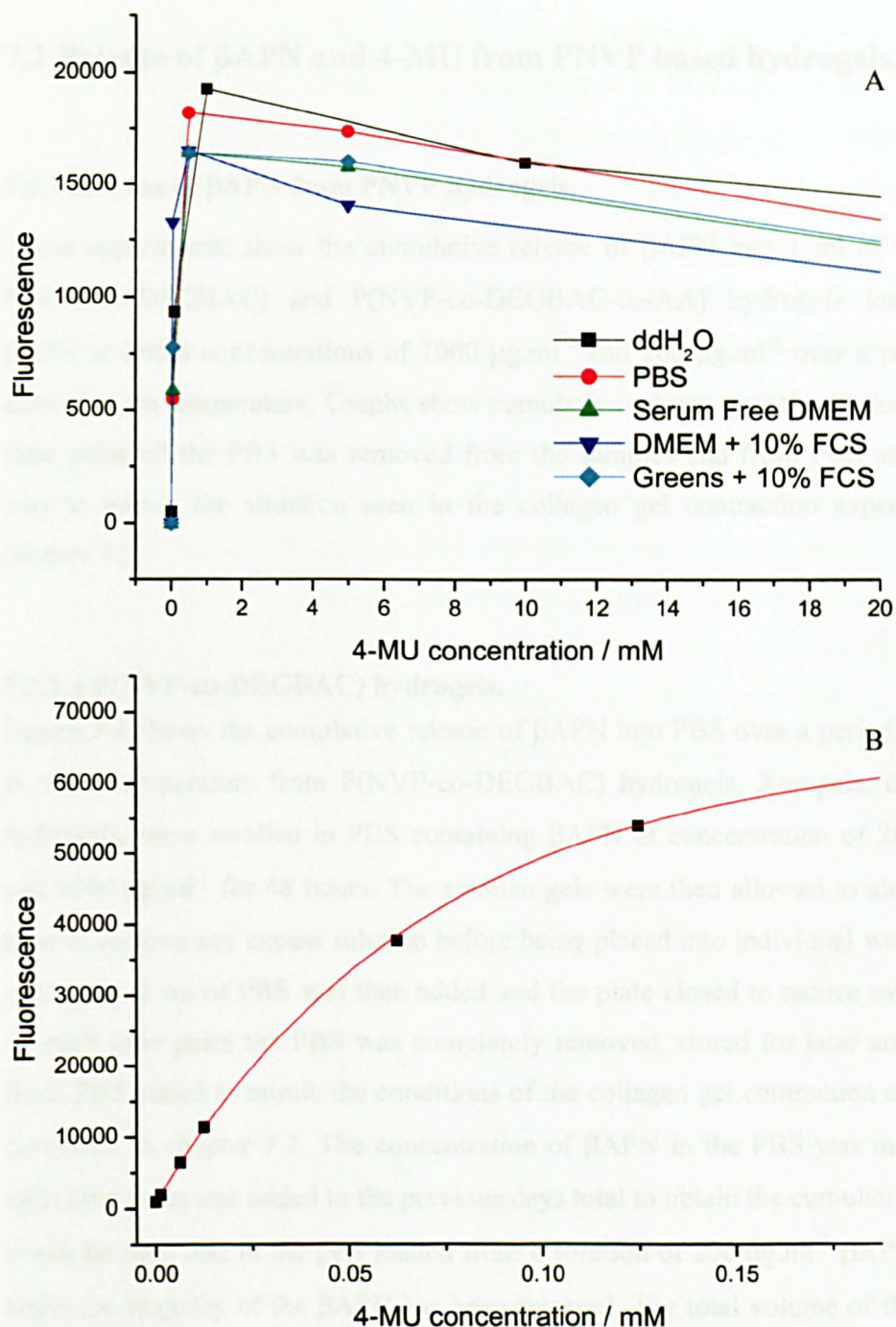
Figure 7.3 Calibration curves for A) 4-MU dissolved in a variety of different solvents. B) Calibration curve used for drug release experiments. Excitation wavelength 360 nm, emission monitored at 440/460 nm. Data expressed as mean ± SEM of n=6 samples.

7.2 Release of βAPN and 4-MU from PNVP based hydrogels.

7.2.1 Release of βAPN from PNVP hydrogels.

These experiments show the cumulative release of βAPN into 1 ml of PBS from P(NVP-co-DEGBAC) and P(NVP-co-DEGBAC-co-AA) hydrogels loaded with βAPN at initial concentrations of 1000 μg.ml⁻¹ and 200 μg.ml⁻¹ over a period of 6 days at room temperature. Graphs show cumulative release over this period. At each time point all the PBS was removed from the samples and fresh PBS added. This was to mimic the situation seen in the collagen gel contraction experiments in chapter 7.3.

7.2.1.1 P(NVP-co-DEGBAC) hydrogels.

Figure 7.4 shows the cumulative release of βAPN into PBS over a period of 6 days at room temperature from P(NVP-co-DEGBAC) hydrogels. Xerogels, dehydrated hydrogels, were swollen in PBS containing βAPN at concentration of 200 μg.ml⁻¹ and 1000 μg.ml⁻¹ for 48 hours. The swollen gels were then allowed to air dry for 1 hour to remove any excess solution before being placed into individual wells of a 48 well plate. 1 ml of PBS was then added and the plate closed to reduce evaporation. At each time point the PBS was completely removed, stored for later analysis and fresh PBS added to mimic the conditions of the collagen gel contraction experiment described in chapter 7.3. The concentration of βAPN in the PBS was measured at each time point and added to the previous days total to obtain the cumulative release. It can be seen that in the gels loaded from a solution of 200 μg.ml⁻¹ βAPN after 48 hours the majority of the βAPN has been released. The total volume of the swollen gel is approximately 500 μl and therefore it is important to note that the concentration of the final solution would not be the same as the concentration of the loading solution i.e. a final cumulative concentration of 200 μg.ml⁻¹ would not be expected. If all the βAPN is being released from a gel loaded from 200 μg.ml⁻¹ a maximum achievable concentration would be approximately 100 μg.ml⁻¹ which is close to the value of 70 μg.ml⁻¹ seen in figure 7.4. The gel loaded from 1000 μg.ml⁻¹ could potentially achieve a maximum concentration of approximately 500 μg.ml⁻¹

however this is not seen in figure 7.4 with the maximum concentration of 160 μg.ml^{-1} being obtained. Again most of the drug appears to be released over the first 48 hours of the experiment. This is further illustrated by the fractional release graphs shown in figure 7.5. Figure 7.5.A shows that approximately 80% of the βAPN is released within the first 48 hours from the P(NVP-co-DEGBAC) hydrogel loaded from a 200 μg.ml^{-1} solution. Figure 7.5.B shows that a similar quantity of the βAPN was also released within the first 48 hours from the P(NVP-co-DEGBAC) hydrogel loaded from a 1000 μg.ml^{-1} solution. The fractional release data have been fitted with curves of the form $y=kx^n$. This allows the rate constant (k) and diffusional exponent (n) to be calculated. Both of these curves are a fairly good fit with R^2 values of 0.844 and 0.791 respectively. The diffusional exponents derived from these graphs, 0.233 ± 0.054 and 0.186 ± 0.023 respectively are much lower than the value of 0.5 generally expected for Fickian release or the value of 0.45 expected for Fickian release from cylindrical samples. They are close to the values of 0.13, 0.19 and 0.23 obtained for the release of 5-FU from PNVP based hydrogels by Liu et al. (2002b).
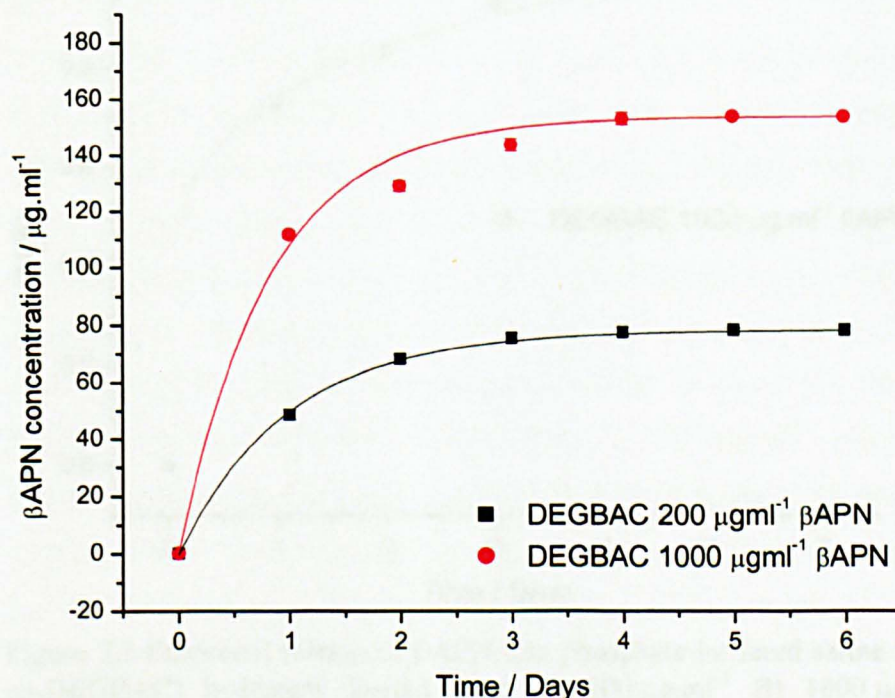
Figure 7.4 Release of βAPN into phosphate buffered saline from P(NVP-co-DEGBAC) hydrogels over a period of 6 days at room temperature. After reaction with TNBS for 2 hours at room temperature absorbance was read at 340 nm and referenced at 630 nm. Data expressed as mean ± SEM of n=6 samples.
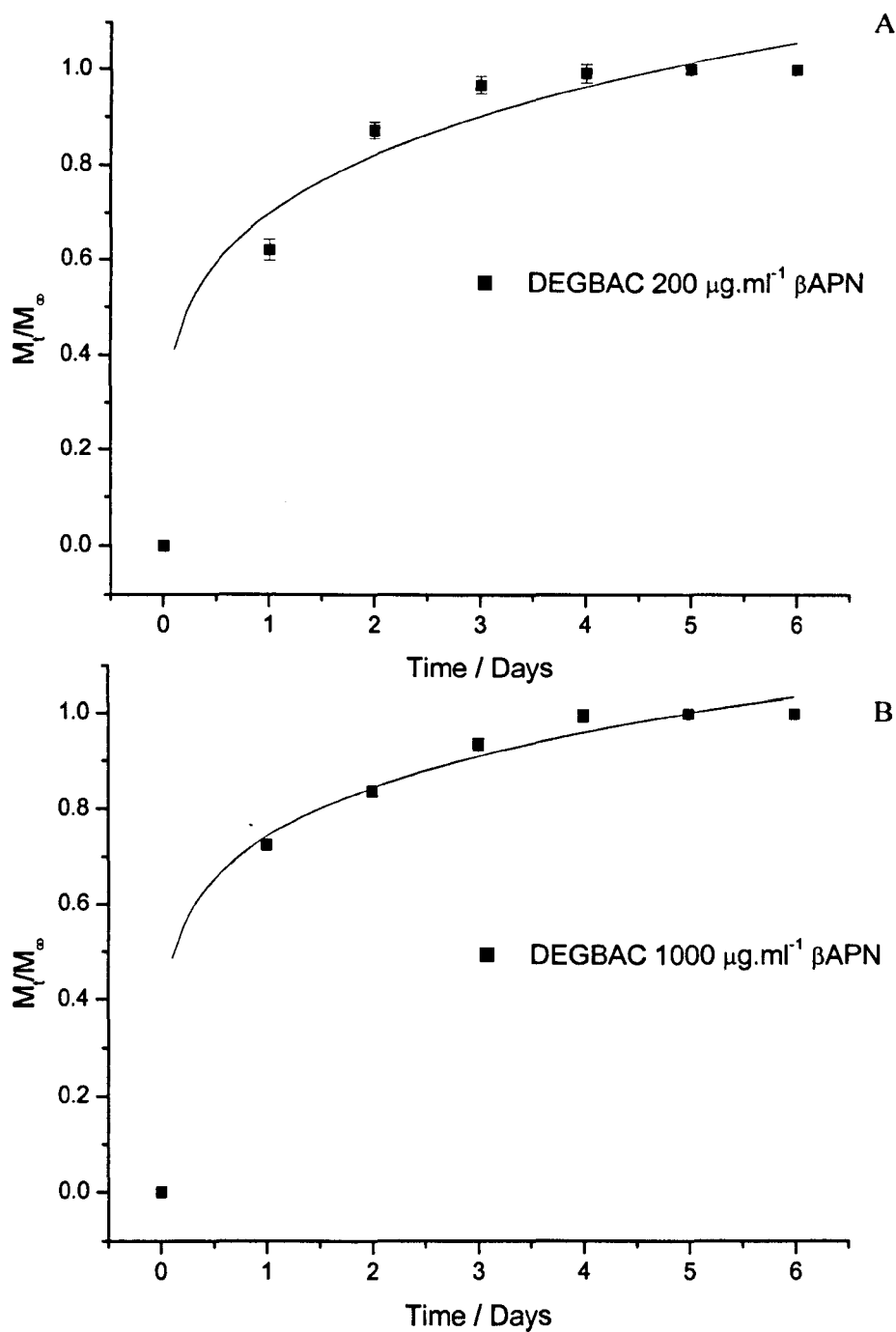
Figure 7.5 Fractional release of βAPN into phosphate buffered saline from P(NVP-co-DEGBAC) hydrogels loaded from A) 200 μg.ml^{-1} B) 1000 μg.ml^{-1} βAPN solutions over a period of 6 days at room temperature. Fractional release plotted from release data used for figure 7.4 plotted as concentration at time t / final concentration. Data expressed as mean ± SEM of n=6 samples.

7.2.1.2 PNVP-co-DEGBAC-co-AA hydrogels.

Figure 7.6 shows the cumulative release of βAPN into PBS over a period of 6 days at room temperature from P(NVP-co-DEGBAC-co-AA) hydrogels. Xerogels were swollen in PBS containing βAPN at concentrations of 200 $\mu g.ml^{-1}$ and 1000 $\mu g.ml^{-1}$ for 48 hours. The swollen gels were then allowed to air dry for 1 hour to remove any excess solution before being placed into individual wells of a 48 well plate. 1 ml of PBS was then added and the plate closed to reduce evaporation. At each time point the PBS was completely removed, stored for later analysis and fresh added to mimic the conditions of the collagen gel contraction experiment described in chapter 7.3. The concentration of βAPN in the PBS was measured at each time point and added to the previous days total to obtain the cumulative release. It can be seen that in the gels loaded from a solution of 200 $\mu g.ml^{-1}$ βAPN after 24 hours the majority of the βAPN has been released. Again it is important to note that the concentration of the final solution would not be the same as the concentration of the loading solution i.e. a final cumulative concentration of 200 $\mu g.ml^{-1}$ would not be expected. If all the βAPN is being released from a gel loaded from 200 $\mu g.ml^{-1}$ a maximum achievable concentration would be approximately 100 $\mu g.ml^{-1}$ which is again close to the value of 70 $\mu g.ml^{-1}$ seen in figure 7.6. The gel loaded from 1000 $\mu g.ml^{-1}$ could potentially achieve a maximum concentration of approximately 500 $\mu g ml^{-1}$ however once again this is not seen in figure 7.6 with the maximum concentration of 150 $\mu g.ml^{-1}$ being obtained. In this case as with the P(NVP-co-DEGBAC) gels most of the drug appears to be released over the first 48 hours of the experiment. This is further illustrated by the fractional release graphs shown in figure 7.7. The P(NVP-co-DEGBAC-co-AA) hydrogel loaded from a 200 $\mu g.ml^{-1}$ solution releases approximately 70% of the βAPN within the first 24 hours as illustrated in figure 7.7.A. Figure 7.7.B shows that approximately 80% of the βAPN is released within the first 24 hours from the P(NVP-co-DEGBAC-co-AA) hydrogel loaded from a 1000 $\mu g.ml^{-1}$ solution. The fractional release profiles obtained from the P(NVP-co-DEGBAC-co-AA) hydrogels loaded from 200 and 1000 $\mu g.ml^{-1}$ solutions of βAPN give diffusional exponents of 0.171 ± 0.05 and 0.133 ± 0.02 respectively. These curves have R^2 values of 0.791 and 0.927 respectively. These values again are much lower than the value of 0.5 generally expected for Fickian release or the value of

0.45 expected for Fickian release from cylindrical samples. Again, they are close to the values of 0.13, 0.19 and 0.23 obtained for the release of 5-FU from PNVP based hydrogels by Liu et al. (2002b). The addition of the acrylic acid to the P(NVP-co-DEGBAC) hydrogel did alter the diffusion exponents. The exponents obtained for the P(NVP-co-DEGBAC-co-AA) hydrogels releasing βAPN were reduced compared to the exponents for the P(NVP-co-DEGBAC) hydrogels releasing βAPN from both loading solutions.

Figure 7.6 Release of βAPN into phosphate buffered saline from P(NVP-co-DEGBAC-co-AA) hydrogels over a period of 6 days at room temperature. After reaction with TNBS for 2 hours at room temperature absorbance was read at 340 nm and referenced at 630 nm n=6.

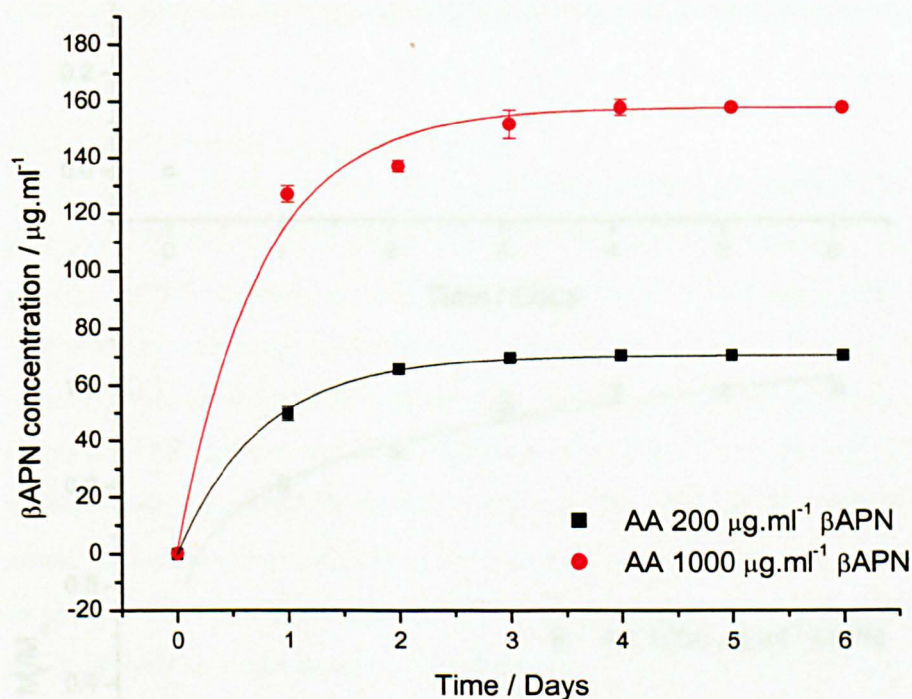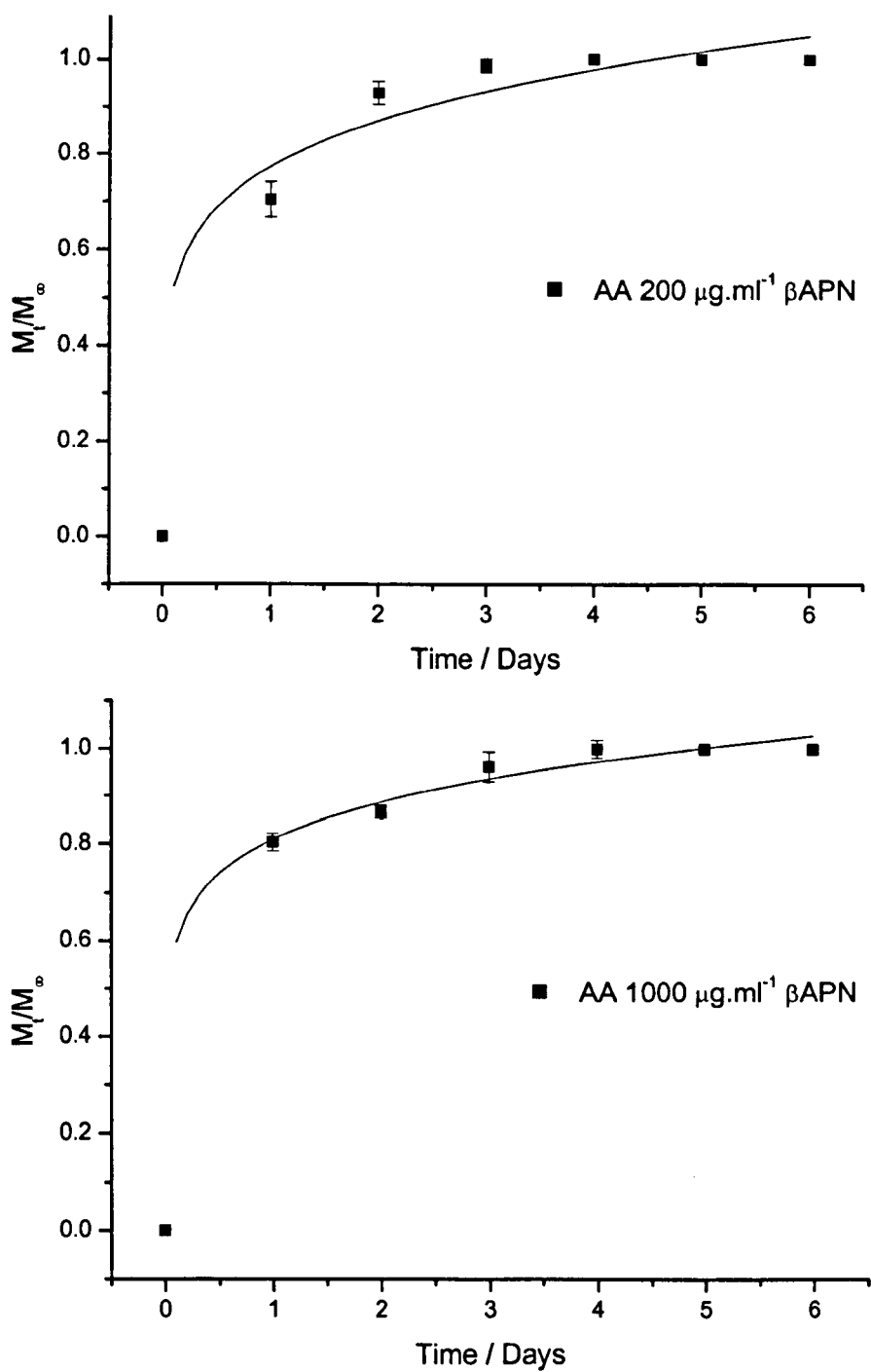Figure 7.7 Fractional release of βAPN into phosphate buffered saline from P(NVP-co-DEGBAC-co-AA) hydrogels over a period of 6 days at room temperature. Fractional release plotted from release data used for figure 7.6 plotted as concentration at time t / final concentration.

7.2.2 Release of 4-MU from PNVP hydrogels.

These experiments show the cumulative release of 4-MU into phosphate buffered saline from P(NVP-co-DEGBAC) and P(NVP-co-DEGBAC-co-AA) hydrogels loaded with 4-MU at initial concentrations of 1 mM or 4 mM over a period of 6 days at room temperature. Graphs show cumulative release over this period. At each time point all the PBS was removed from the samples and fresh PBS added. This was to mimic the situation seen in the collagen gel contraction experiments in chapter 7.3.

7.2.2.1 PNVP-co-DEGBAC hydrogels.

Figure 7.8 shows release of 4-MU into Greens medium containing 10% FCS over a period of 6 days at 37°C from P(NVP-co-DEGBAC) hydrogels. Xerogels were swollen in PBS containing 4-MU at concentration of 1 mM and 4 mM for 48 hours. The swollen gels were then allowed to air dry in sterile conditions for 1 hour to remove any excess solution before being placed into individual wells of a 48 well plate. 1 ml of medium was then added and the plate closed. At each time point the medium was completely removed and stored for later analysis and fresh medium added to mimic the conditions of the collagen gel contraction experiment described in chapter 7.3. The concentration of 4-MU in the PBS was measured at each time point and added to the previous days total to obtain the cumulative release. It can be seen that in the gels loaded from a solution of 1 mM 4-MU after 24 hours the majority of the 4-MU has been released. Again it is important to note that the concentration of the final solution would not be the same as the concentration of the loading solution i.e. a final cumulative concentration of 1 mM would not be expected. If all the 4-MU is being released from a gel loaded from 1 mM solution a maximum achievable concentration would be approximately 0.5 mM which is near to the value of 0.3 mM seen in figure 7.8. The gel loaded from 4 mM could potentially achieve a maximum concentration of approximately 2 mM however figure 7.8 shows a value of approximately 1.5 mM is obtained. In this case as with the gels loaded from a 1 mM solution most of the drug appears to be released over the first 24 hours of the experiment. This is further illustrated by the fractional release graphs shown in figure 7.9. Figure 7.9.A shows that approximately 80% of the 4-MU is released within the first 24 hours from the P(NVP-co-DEGBAC)

hydrogel loaded from a 1 mM 4-MU solution. Figure 7.9.B shows that a similar quantity of the 4-MU was also released within the first 24 hours from the P(NVP-co-DEGBAC) hydrogel loaded from a 4mM 4-MU solution. Both of these curves are not a good fit with R^2 values of 0.682 and 0.730 respectively. The diffusional exponents derived from these graphs, 0.074 ± 0.03 and 0.107 ± 0.03 respectively are indicative of non Fickian release. These values again are much lower than the value of 0.5 generally expected for Fickian release or the value of 0.45 expected for Fickian release from cylindrical samples. Again, they are close to the values of 0.13, 0.19 and 0.23 obtained for the release of 5-FU from PNVP based hydrogels by Liu et al. (2002b).
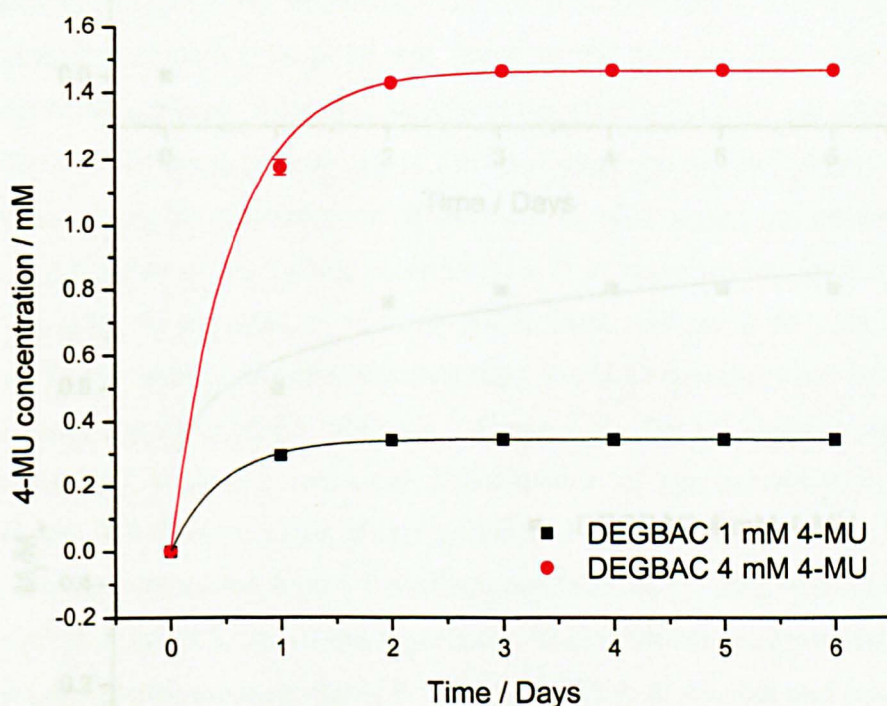
Figure 7.8 Release of 4-MU into Greens medium from P(NVP-co-DEGBAC) hydrogels over a period of 6 days at 37°C. Fluorescence was measured using excitation of 360 nm and measuring emission at 440/460 nm n=6.
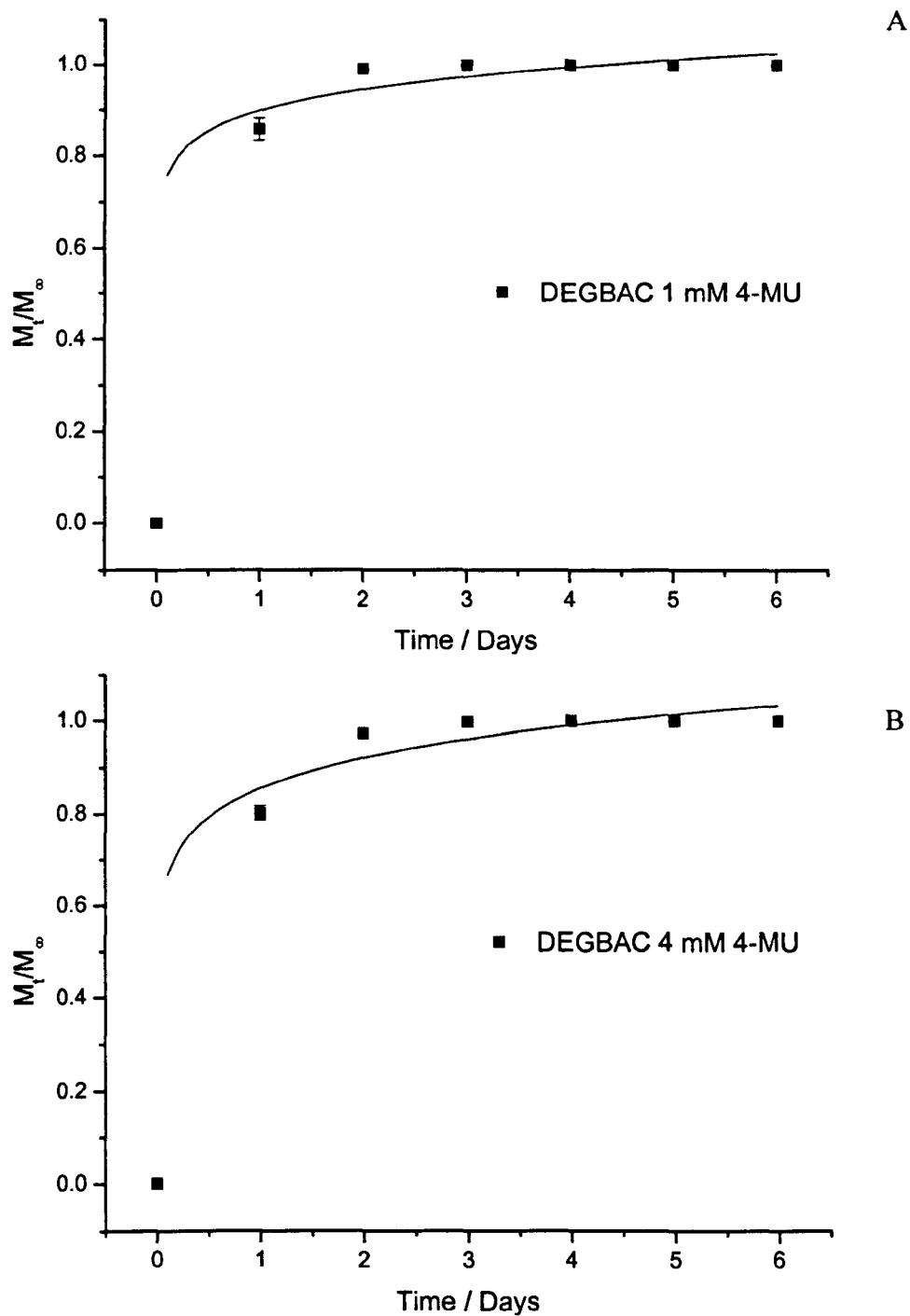
Figure 7.9 Fractional release of 4-MU into Greens medium from P(NVP-co-DEGBAC) hydrogels over a period of 6 days at 37°C. Fractional release plotted from release data used for figure 7.8 plotted as concentration at time t / final concentration.

7.2.2.2 PNVP-co-DEGBAC-co-AA hydrogels.

Figure 7.10 shows the cumulative release of 4-MU into Greens medium containing 10% FCS over a period of 6 days at $37^{\circ}C$ from P(NVP-co-DEGBAC-co-AA) hydrogels. Xerogels were swollen in PBS containing 4-MU at concentration of 1 mM and 4 mM for 48 hours. The swollen gels were then allowed to air dry for 1 hour in sterile conditions to remove any excess solution before being placed into individual wells of a 48 well plate. 1 ml of medium was then added and the plate closed. At each time point the medium was completely removed and stored for later analysis and fresh added to mimic the conditions of the collagen gel contraction experiment described in section 7.3. The concentration of 4-MU in the PBS was measured at each time point and added to the previous days total to obtain the cumulative release. It can be seen that in the gels loaded from a solution of 1 mM 4-MU after 24 hours the majority of the 4-MU has been released. Again it is important to note that the concentration of the final solution would not be the same as the concentration of the loading solution i.e. a final cumulative concentration of 1 mM would not be expected. If all the 4-MU is being released from a gel loaded from 1 mM a maximum achievable concentration would be approximately 0.5 mM which is close to the value of 0.3 mM seen in figure 7.10. The gel loaded from 4 mM could potentially achieve a maximum concentration of approximately 2 mM however figure 7.10 shows a value of approximately 1 mM 4MU is obtained. In this case as with the gels loaded from a 1 mM solution most of the drug appears to be released over the first 24 hours of the experiment. This is further illustrated by the fractional release graphs shown in figure 7.11. Figure 7.11.A shows that approximately 80% of the 4-MU is released within the first 24 hours from the P(NVP-co-DEGBAC) hydrogel loaded from a 1 mM 4-MU solution. Figure 7.11.B shows that a similar quantity of the 4-MU was also released within the first 24 hours from the P(NVP-co-DEGBAC) hydrogel loaded from a 4 mM 4-MU solution. Again both of these curves are not a good fit with R^2 values of 0.676 and 0.716 respectively. The diffusional exponents derived from these graphs, 0.074 ± 0.03 and 0.101 ± 0.03 respectively are indicative of non-Fickian release. Again these values are close to those obtained for the release of 5-FU from PNVP hydrogels (Liu and Rimmer 2002b). The addition of the acrylic acid to the P(NVP-co-DEGBAC) hydrogel had no effect on the release of the 4-MU.
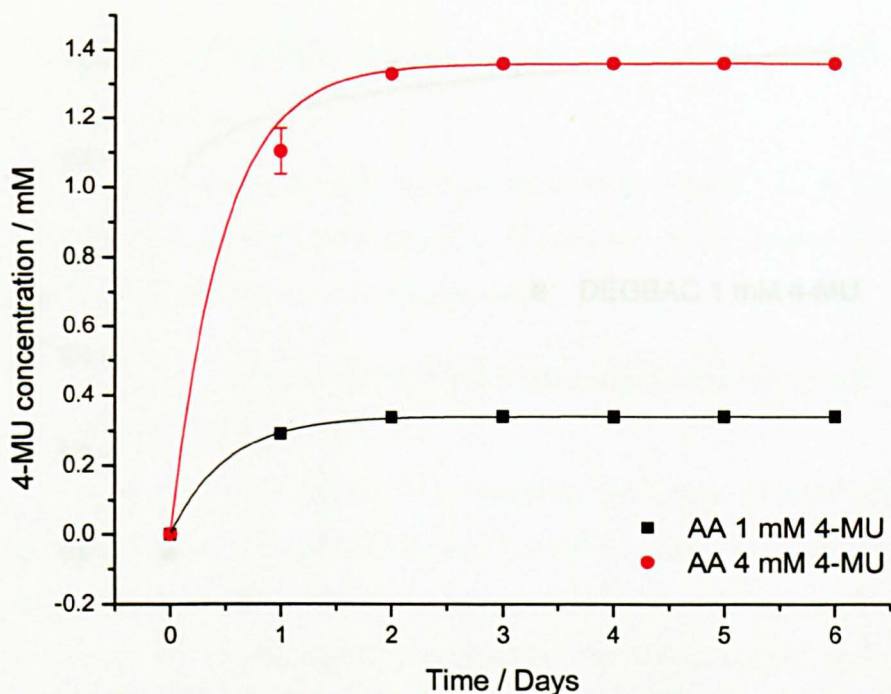
Figure 7.10 Release of 4-MU into phosphate buffered saline from P(NVP-co-DEGBAC-co-AA) hydrogels over a period of 6 days at room temperature. Fluorescence was measured using excitation of 360 nm and measuring emission at 440/460 nm n=6.
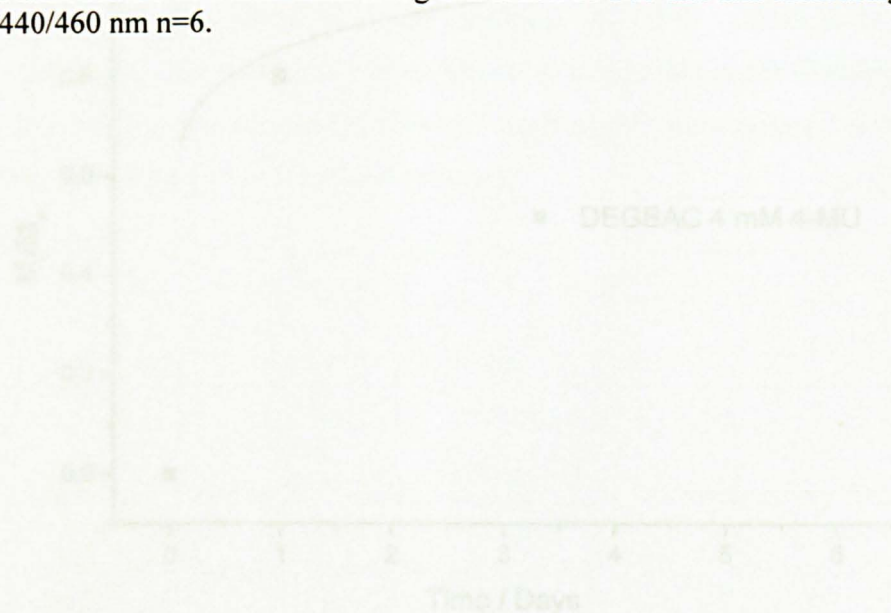
Figure 7.11 Fractional release of 4-MU into Greens medium from P(NVP-co-DEGBAC-co-AA) hydrogels over a period of 6 days at 37°C. Fractional release plotted from release data used for figure 7.10 plotted as concentration at time t / final concentration.

7.3 Effect of PNVP hydrogels loaded with βAPN or 4MU on collagen gel contraction.

In these experiments the gels were prepared as per chapter 2.7.6. Even though the βAPN had no effect on the contraction of collagen gels in chapter 6.1 βAPN loaded gels were still included in these experiments.

7.3.1 Effect of P(NVP-co-DEGBAC) hydrogels loaded with βAPN or 4-MU on contraction of collagen gels.

The graph shown in figure 7.12 illustrates the effect of P(NVP-co-DEGBAC) hydrogels loaded with βAPN and 4-MU on the contraction of collagen gels over a period of 6 days. The initial contraction seen in the first 24 hours is thought in part to be due to the innate elastic properties of the collagen gels with only a small contribution from the cells. All of the treated collagen gels contracted significantly less over the initial 24 hours than the control. Over the following 24 hours only the hydrogel loaded from a 4 mM solution of 4-MU significantly reduced the contraction. The hydrogels had no effect on contraction at any further time points. It is interesting to note that the hydrogel itself significantly reduced contraction in the absence of drug over the initial 24 hours.

Figure 7.12 Effect of βAPN and 4-MU loaded P(NVP-co-DEGBAC) hydrogels on contraction of co-cultured collagen gels over 6 days. Results shown are mean ± SEM of n=3 experiments (3 replicates per experiment). * indicates a significant difference in area from untreated collagen gels (1-way ANOVA + Bonferroni correction). *p<0.05 **p<0.01 ***p<0.001.

7.3.2 Effect of P(NVP-co-DEGBAC-co-AA) hydrogels loaded with βAPN or 4-MU on contraction of collagen gels.

The graph shown in figure 7.13 illustrates the effect of P(NVP-co-DEGBAC-co-AA) hydrogels loaded with βAPN and 4-MU on the contraction of collagen gels over a period of 6 days. The initial contraction seen in the first 24 hours is thought in part to be due to the innate elastic properties of the collagen gels with only a small contribution from the cells. Unlike the P(NVP-co-DEGBAC) hydrogels the P(NVP-co-DEGBAC-co-AA) hydrogels have no significant effect on the contraction of collagen gels by themselves. The hydrogels loaded with βAPN from a 1000 µg.ml^{-1} solution significantly reduce contraction during the first 24 hours as do the hydrogels loaded from a 1 mM solution of 4-MU. The hydrogel loaded from a 4 mM solution of 4-MU significantly reduced the contraction of the collagen gels compared to the untreated gels up to day 2.
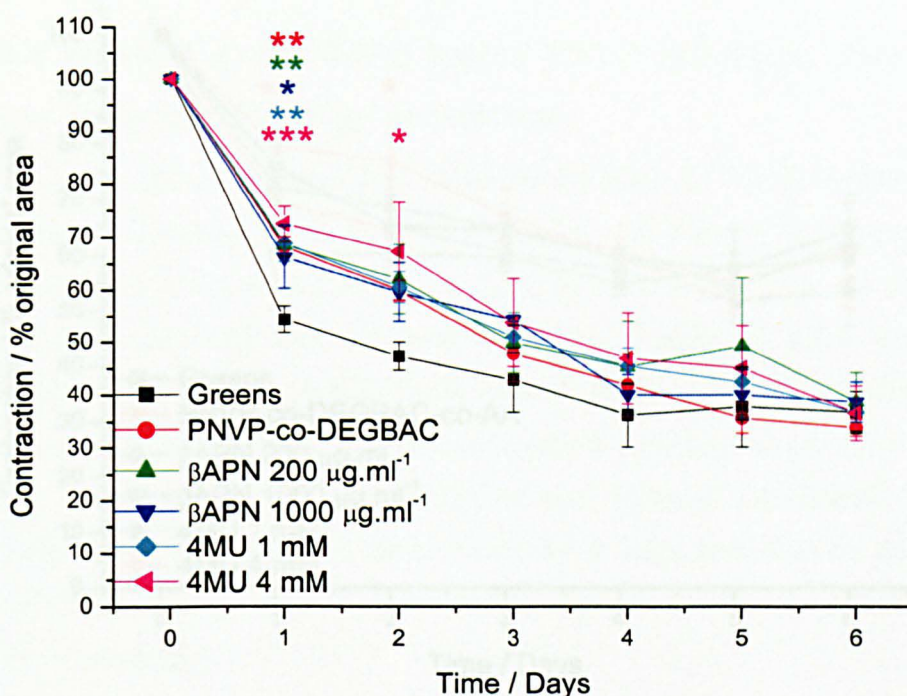
Figure 7.13 Effect of βAPN and 4-MU loaded P(NVP-co-DEGBAC) hydrogels on contraction of co-cultured collagen gels over 6 days. Results shown are mean ± SEM of n=3 experiments (3 replicates per experiment) * indicates significant difference in area from untreated collagen gels (1-way ANOVA + Bonferroni correction). *p<0.05 **p<0.01 ***p<0.001.

7.4 Effect of 4 mM 4-MU loaded PNVP hydrogels, changed every two days, on collagen gel contraction.

The graph shown in figure 7.14 illustrates the effect of P(NVP-co-DEGBAC) and P(NVP-co-DEGBAC-co-AA) hydrogels loaded with 4 mM 4-MU on the contraction of collagen gels over a period of 6 days. In these experiments the hydrogel was removed every two days and fresh 4 mM 4-MU hydrogel added. Replacing the P(NVP-co-DEGBAC) hydrogel with fresh 4 mM 4-MU hydrogel every two days significantly reduces the contraction compared to the untreated control over the full 6 day period. The P(NVP-co-DEGBAC-co-AA) hydrogel significantly reduces the contraction over 5 of the 6 days. However the large error bars on day 6 prevent significance being obtained. There was no significant difference observed between the two hydrogels.
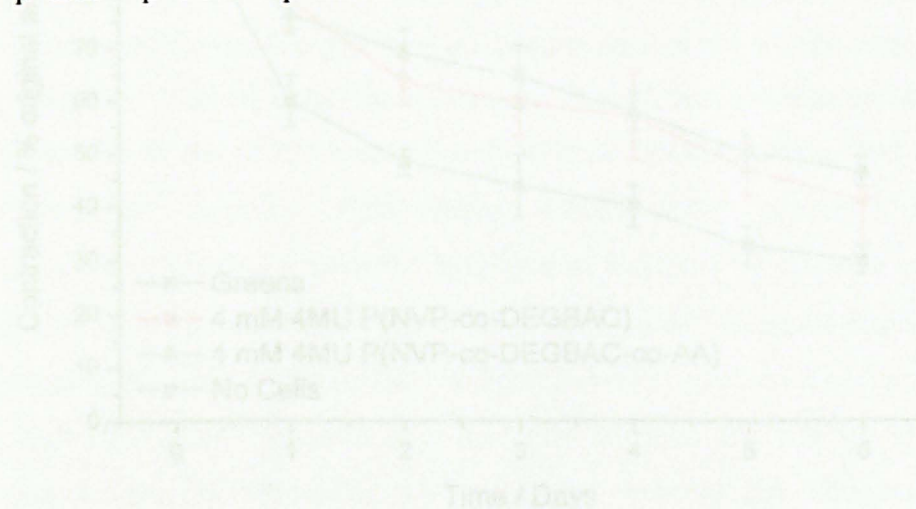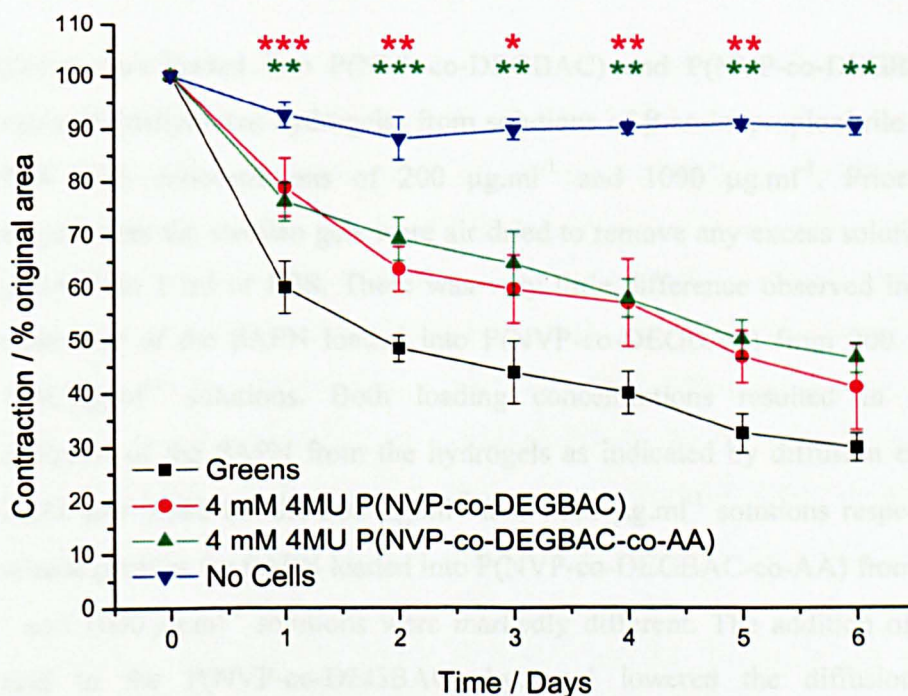

Figure 7.14 Effect of PNVP based hydrogels loaded with 4 mM 4-MU on contraction of co-cultured collagen gels over 6 days. Hydrogels changed every 2 days. Results shown are mean ± SEM of n=3 experiments (3 replicates per experiment) * indicates significant difference in area from untreated collagen gels (2-way ANOVA + Bonferroni correction). *p<0.05 **p<0.01 ***p<0.001.

7.5 Summary of results.

After the synthesis and characterisation of the hydrogels the evaluation of the 4-MU and βAPN drug release profiles were obtained. The TNBS (2,4,6-trinitrobenzenesulfonic acid) assay, an assay for detection and quantification of primary amines in peptide solutions was modified to allow detection and quantification of βAPN in phosphate buffered saline. This is not ideal but it was not possible to detect the βAPN in cell culture media containing solutions. This was thought to be due to the presence of amino acids in the media and proteins in the foetal calf serum. The 4-MU was detected using fluorescence and could be accurately detected in various media, water, PBS, PBS + 10% FCS, serum free DMEM, DMEM + 10% FCS and Greens medium + 10% FCS.

βAPN was loaded into P(NVP-co-DEGBAC) and P(NVP-co-DEGBAC-co-AA) xerogels (dehydrated hydrogels) from solutions of β-aminopropionitrile fumarate in PBS with concentrations of 200 μg.ml^{-1} and 1000 μg.ml^{-1}. Prior to release experiments the swollen gels were air dried to remove any excess solution and then placed into 1 ml of PBS. There was very little difference observed in the release behaviour of the βAPN loaded into P(NVP-co-DEGBAC) from 200 μg.ml^{-1} and 1000 μg.ml^{-1} solutions. Both loading concentrations resulted in non-Fickian diffusion of the βAPN from the hydrogels as indicated by diffusion exponents of 0.231 and 0.186 for the 200 μg.ml^{-1} and 1000 μg.ml^{-1} solutions respectively. The release profiles for βAPN loaded into P(NVP-co-DEGBAC-co-AA) from 200 μg.ml^{-1} and 1000 μg.ml^{-1} solutions were markedly different. The addition of the acrylic acid to the P(NVP-co-DEGBAC) hydrogel lowered the diffusion exponent. Diffusion exponents of 0.171 and 0.133 were calculated for the release of the βAPN from the P(NVP-co-DEGBAC-co-AA) hydrogels loaded from 200 μg.ml^{-1} and 1000 μg.ml^{-1} solutions of 3-aminopropionitrile fumarate.

4-MU was loaded into P(NVP-co-DEGBAC) and P(NVP-co-DEGBAC-co-AA) xerogels from solutions of 4-methyl umbelliferone sodium salt in PBS with concentrations of 1 mM and 4 mM. Prior to release experiments the swollen gels

were air dried to remove any excess solution and then placed into 1 ml of PBS. There was very little difference observed in the release profiles for the P(NVP-co-DEGBAC) hydrogels loaded from the 1 mM and 4 mM 4-MU solutions. The release profiles calculated for these hydrogels gave diffusional exponents of 0.074 and 0.107 for the 1 mM and 4 mM loaded hydrogels. With both hydrogels an initial burst release was observed during the first 24 hours where 80% of the 4-MU was released. The hydrogel loaded from the 1 mM solution released the remaining 20% of 4-MU over the following 48 hours. Similarly the hydrogel loaded from the 4 mM solution released the remaining 20% of 4-MU over the following 24 hours. The addition of the acrylic acid to the P(NVP-co-DEGBAC) hydrogel had no effect on the release of the 4-MU. Diffusional exponents calculated for the release of the 4-MU from the 1 mM and 4 mM P(NVP-co-DEGBAC-co-AA) hydrogel were 0.074 and 0.101 respectively.

Table 7.1 summarises the diffusion exponents calculated for the release of βAPN and 4-MU from the PNVP based hydrogels. The acrylic acid was added to the P(NVP-co-DEGBAC) hydrogel in an attempt to modify the release of the βAPN from the hydrogel. The calculated diffusion exponents show that adding the acrylic acid to the hydrogel reduced the diffusion exponent. The addition of the acrylic acid to the P(NVP-co-DEGBAC) hydrogel has no effect on the calculated diffusion exponents for the release of the 4-MU. All of the diffusional exponents are in the same range as those observed by Liu et al. (2002b) for the release of 5-fluorouracil from PNVP based hydrogels. However the 5-FU was part of the crosslinker used in the hydrogel synthesis. Its release was therefore governed not only by diffusion but by crosslinker degradation (Liu and Rimmer 2002b). It was not possible to physically incorporate the βAPN or 4-MU into the hydrogels and therefore the release mechanism despite the similarities in diffusion exponents cannot be the same.

Table 7.1 Summary of diffusion exponents

		P(NVP-co-DEGBAC)	P(NVP-co-DEGBAC-co-AA)
βAPN / μgml^{-1}	200	0.232 ± 0.05	0.171 ± 0.05
	1000	0.186 ± 0.02	0.133 ± 0.02
4-MU / mM	1	0.074 ± 0.03	0.073 ± 0.03
	4	0.107 ± 0.03	0.101 ± 0.03

Collagen gels were then used to evaluate the effects of these drug loaded hydrogels on contraction. The hydrogels examined were P(NVP-co-DEGBAC) and P(NVP-co-DEGBAC-co-AA) hydrogels loaded with βAPN from solutions of 200 μg.ml^{-1} and 1000 μg.ml^{-1} and 4-MU from solutions of 1 mM and 4 mM. Hydrogels swollen in PBS in the absence of the βAPN and 4-MU were used as controls. The P(NVP-co-DEGBAC) hydrogels loaded from a 4 mM solution of 4-MU produced a significant decrease in the contraction of the collagen gels up to day 2 of the experiment. In all cases the contraction of the collagen gels treated with the P(NVP-co-DEGBAC) hydrogel was significantly reduced at the 1 day time point. This was unexpected as the βAPN loaded hydrogels appeared to reduce contraction whilst βAPN by itself had no effect on the collagen gels. The P(NVP-co-DEGBAC-co-AA) hydrogels, loaded from a 1000 μg.ml^{-1} solution of βAPN and 1 mM solution of 4MU produced a significant decrease in the contraction of the collagen gels up to day 1 of the experiment. However the P(NVP-co-DEGBAC-co-AA) hydrogel loaded from a 4 mM solution of 4-MU produced a significant decrease in the contraction of the collagen gels up to day 2.

Reduction in collagen gel contraction was only observed up to day 2 when treated with any combination of hydrogel and anti-contraction agent. The hydrogels loaded from solutions of 4 mM 4-MU however appeared to be the most promising. It was therefore proposed to change the hydrogel every two days. Again all the media was removed at each 24 hour time point. It should be noted that the cells in the collagen gels will not be exposed to a constant concentration of 4-MU. It was shown in figures 7.9 and 7.11 that approximately 80% of the 4-MU is released from both the P(NVP-co-DEGBAC) and P(NVP-co-DEGBAC-co-AA) hydrogels with most of the remaining 4-MU released over the following 24 hours. This means that on the day

when the hydrogels are changed the cells will be exposed to a much higher concentration of 4-MU than on the days where the hydrogels are not changed. Changing the hydrogel every two days resulted in a significant reduction in collagen gel contraction when the gels were treated with both the P(NVP-co-DEGBAC) and the P(NVP-co-DEGBAC-co-AA) hydrogels loaded from a 4 mM 4-MU solution at the majority of the time points up to 6 days.

8. Discussion.

The overall aim of this project was to develop a novel poly(N-vinylpyrrolidinone) (PNVP) based hydrogel wound dressing which could be used to release anti-contraction agents to prevent or reduce skin graft contracture.

To achieve this a series of specific objectives were identified 1) to evaluate the material properties and cellular toxicity of novel PNVP hydrogels, 2) To examine the cytotoxicity of the identified anti contraction agents, βAPN and 4-MU, 3) to determine whether βAPN and 4-MU could reduce contraction in a collagen gel model, 4) to determine if 4-MU could prevent or reduce contraction in the reconstructed skin model, 5) to examine the loading and release of βAPN and 4-MU into and from PNVP hydrogels and 6) to examine to what extent the drug loaded hydrogels would reduce the contraction of the collagen gel and reconstructed skin models.

A novel PNVP hydrogel was produced with material properties suitable for use as a wound dressing. Cytotoxicity testing showed that whilst cells could not be grown on the hydrogel this hydrogel was not cytotoxic. If human dermal fibroblasts were grown in indirect contact with the hydrogel then cell viability could be stimulated. Cytotoxicity testing of βAPN showed that this drug at the concentrations identified was also not cytotoxic. βAPN also had no affect on keratinocyte proliferation or differentiation at the concentrations identified. Cytotoxicity testing of 4-MU showed that this drug over the concentrations examined could significantly reduce cell viability and keratinocyte proliferation and differentiation. Despite or perhaps because of these cytotoxic effects 4-MU significantly reduced the contraction of both collagen I gels and the reconstructed skin model. βAPN was not shown to have any effect on the contraction of collagen I gels but previous work in this laboratory had shown βAPN to be effective in reducing the contraction of the reconstructed skin model. The water soluble salts of βAPN and 4-MU were loaded into the PNVP hydrogels and the release of the drugs from the hydrogels monitored. The drugs were released from the hydrogels in a predictable manner over a 48 hour period. The 4-

MU loaded hydrogels were then shown to significantly reduce the contraction of collagen I gels when the hydrogels were changed every two days.

In this thesis initially attempts were made to produce PNVP oligomers with differing functionalities. This was in an attempt to produce PNVP polymers with a greater range of applications. Unfortunately attempts to produce functionalised NVP oligomers had low yields and these synthesis routes proved impractical for the large quantities needed for hydrogel synthesis. Therefore PNVP hydrogel membranes crosslinked with either ethyleneglycol dimethacrylate (EGDMA) or diethyleneglycol bisallylcarbonate (DEGBAC) were synthesised and their material and biological properties evaluated. The P(NVP-co-EGDMA) hydrogels with 1 wt% crosslinker did not have material properties suitable for use as a wound dressing. The polymers, even when synthesised in the absence of solvent, once swollen in phosphate buffered saline (PBS) had an elastic modulus (E) of 0.16 MPa $\pm$ 0.02 MPa and ultimate compressive strength (UCS) of 0.03 MPa with an equilibrium water content (EWC) of 96.2% $\pm$ 0.3%. Increasing the amount of EGDMA in the hydrogel resulted in an opaque, phase separated polymer that disintegrated easily. Increasing the EGDMA content in the polymer to 2 wt% produced a polymer with an EWC of 92.3% $\pm$ 0.6%. Unfortunately it was not possible to get an elastic modulus or ultimate compressive strength for this polymer. Changing the crosslinker to DEGBAC reduced the EWC to 92.2% $\pm$ 0.1% and gave an E = 0.22 MPa $\pm$ 0.02 MPa and UCS=0.29 MPa $\pm$ 0.08 MPa. Once again increasing the DEGBAC concentration in the monomer blend reduced the EWC to 81.7% $\pm$ 0.4% for the 4 wt% DEGBAC hydrogel.

These properties compare favourably with those seen in the literature for hydrogels. The viscoelastic nature of hydrogels makes the experimental determination of their material properties difficult. Values can be calculated for the elastic modulus, ultimate compressive or tensile strength, and the strain at failure. However these values will be dependent on the strain rate and temperature at which the experiment was performed. All experiments performed for this project were performed at a strain rate of 0.1 mm.s^{-1}, this is equivalent to 6 mm.min^{-1}. Cauich-Rodriguez et al. (1996a) produced a PNVP – PVA hydrogel with similar material properties, E = 0.15 MPa $\pm$ 0.07 MPa. Unfortunately it was not possible to obtain published EWC data for these polymers. The hydrogels produced by Hong et al. (1997) do not

have published mechanical data, however data about the EWC's of these polymers have been published. The polymers produced by Hong et al. were primarily synthesised from NVP and the majority of these contained a small amount of HEMA as well as a crosslinking agent (divinyl glycol, diallyl ether, ethyleneglycol dimethacrylate, diethyleneglycol dimethacrylate or tetraethyleneglycol dimethacrylate). All of these polymers had similar EWC's (95.0% - 98.3%) to the P(NVP-co-EGDMA) hydrogels produced for this thesis which had EWC's of 92.3% - 98.3%. Increasing the amount of another hydrophilic monomer, HEMA, in the Hong polymers from 1 wt% to 2 wt% whilst using 0.25 wt% diethyleneglycol dimethacrylate as the crosslinking agent increased the EWC from 95.0% to 98.3%. The P(NVP-co-DEGBAC) based hydrogels had much lower EWC's (81.7 – 92.2%) than those seen in the Hong et al. polymers. The EWC values seen for the P(NVP-co-DEGBAC) based polymers are similar to those obtained by Lopes et al. (2003) for their PNVP-gelatin interpenetrating networks. The EWC's obtained for these networks ranged from 89.8% for the highest PNVP concentration (70% PNVP : 30% gelatin) to 90.2% for the lowest PNVP concentration (30% PNVP : 70% gelatin). However the PNVP crosslinked network produced as a control had an EWC of 89.9% whilst the crosslinked gelatin control had an EWC of 83%. Potassium persulphate was used to crosslink the PNVP instead of a larger molecule such as EGDMA or DEGBAC and PNVP was used to form the networks instead of the monomer NVP used to produce the polymers in this thesis and the studies mention previously.

Generally, the reactivity ratios for polymerization of NVP and methacrylates do not favour the formation of instantaneous polymer compositions that are similar to the feeds. This can lead to heterogeneous materials. For example the reactivity ratios for copolymerization with furfuryl methacrylate (FM) have been approximated as rFA = 3.92 and rNVP = 0.004 (Zaldivar, Peniche et al. 1993). Copolymerization with glycidyl methacrylate (GMA) gave values of rGMA = 4.32 – 4.53 and rNVP = 0.0075 – 0.0147 (Wen, Xiaonan et al. 1992). The result of this large disparity between the pairs of reactivity ratios means that at high conversion heterogeneous materials contain regions of high crosslink density connected by long linear chains. On the other hand the reactivity ratios obtained by polymerization of α-allyl or β-allyl carbonates are much closer. For example Liu et al. (2000)

determined that for 5-fluoruracil diallylcarbonates that were copolymerized with NVP: $r\alpha$-allyl = 0.32 and rNVP = 0.97 and $r\beta$-allyl = 0.61 and rNVP = 1.3 (Liu and Rimmer 2002b). Therefore, the use of a bisallylcarbonate as a crosslinker in the synthesis of hydrogels, by the radical polymerization of NVP should lead to materials with more homogeneous distributions of crosslinks. Clearly changes in the distribution of crosslinks have a large effect on the properties i.e. EWC, E and UCS, of the hydrogels.

Literature reports (Bruining, Koole et al. 1999) suggested that crosslinking the PNVP with a carbonate based crosslinker could produce a highly degradable hydrogel which would not be suitable for use as a wound dressing. Bruining et al. (1999) reported the synthesis and degradation of a carbonate crosslinked PNVP based hydrogel. The novel carbonate crosslinker used in the study was much longer than the DEGBAC used in this project and whilst both molecules have two carbonate groups the groups on the novel crosslinker appear to be more accessible and therefore more susceptible to hydrolysis. This may explain why the polymers synthesised by Bruining et al. degraded over time spans varying from under one day to polymers which were still stable after one week in studies performed at $37^{o}C$ in PBS. To ensure that the P(NVP-co-DEGBAC) hydrogels would be stable degradation studies were performed on these hydrogels. EWC measurements were used to monitor hydrogel degradation. If significant degradation was occurring then the EWC would increase. No significant increase in EWC was observed over the 12 days of the experiments. This would suggest that within the limits of the EWC measurements no appreciable degradation was occurring.

Degradable hydrogels are especially useful in drug delivery and tissue engineering applications. Controlling degradation behaviour is one of the critical issues in biomaterials research. Generally biomaterials need to be cleared from the body once they complete their roles in the body, and degradable materials are ideal for this purpose. These polymers are usually viewed as superior materials; however, they have to be approached with caution. The effects of the degradation products on surrounding tissues has to be assessed. Changes in pH associated with the degradation of 'traditional' biodegradable polymers such as poly(lactic acid) or

poly(glycolic acid) can have an adverse effect on the viability of surrounding tissues. The rates of degradation also have to be evaluated as do the effects of degradation on the material properties of the polymer.

The cytotoxicity of these hydrogels was then investigated. Cytotoxicity studies of PNVP based materials reported in the literature shows that PNVP is non cytotoxic and has even been shown to increase cell viability particularly in fibroblasts (Robinson, Sullivan et al. 1990; Vijayasekaran, Chirila et al. 1996; Hong, Chirila et al. 1997; Hong, Chirila et al. 1998; Risbud, Hardikar et al. 2000; Lopes and Felisberti 2003; Sen and Avci 2005; Devine, Devery et al. 2006; Saxena, Mozumdar et al. 2006). However this appears to be the first comprehensive cytotoxicity study using these polymers.

The MTT assay was used to assess the viability of cells cultured in both direct and indirect contact with the hydrogels. Initial studies performed with the P(NVP-co-EGDMA) hydrogel confirmed that PNVP based hydrogels can increase cell viability. Fibroblasts grown in direct contact with the P(NVP-co-EGDMA) polymer did not appear to adhere well to the polymer. Cells were seen either growing on the bottom of the ThinCert™ insert or in small aggregates on the polymer. In both cases the cells stained purple after incubation with MTT indicating that they are viable. The very high water content of this polymer provides a probable explanation for the lack of cell adhesion. For cells to be able to attach successfully to a surface, proteins i.e. fibronectin, laminin or vitronectin first have to adsorb to the surface (Nuttelman, Mortisen et al. 2001). In the case of this polymer the polymer is so highly hydrated that proteins would probably find it difficult to adsorb and therefore the cells would be less likely to attach to the polymers. In experiments where fibroblasts were grown in indirect contact with the P(NVP-co-EGDMA) hydrogel an increase in viability was seen. When fibroblasts were grown in direct contact with the P(NVP-co-DEGBAC) hydrogel there was no increase in fibroblast viability. Again the cells preferred to grow on the bottom of the ThinCert™ insert rather than on the hydrogel. Whilst these cells stained blue when incubated with MTT indicating that they are viable, observations of cell morphology showed that whilst cell attachment to the polymer appears to be increased compared to the P(NVP-co-EGDMA) polymer, fibroblasts do not appear to be able to successfully adhere to the polymer. Again the

high water contents of these polymers may inhibit protein adsorption to the polymer surface and therefore inhibit cell attachment to the polymers. When fibroblasts were grown in indirect contact with the P(NVP-co-DEGBAC) hydrogel a significant increase in fibroblast viability was seen. In all of the experiments where fibroblasts were grown in direct contact with P(NVP-co-DEGBAC-co-AA) hydrogels cell viability was significantly inhibited. Growing fibroblasts in indirect contact with the P(NVP-co-DEGBAC-co-AA) hydrogels had no significant effect on cell viability in all three experiments. Again when the fibroblasts were grown in direct contact with the P(NVP-co-DEGBAC-co-AA) hydrogel the viable fibroblasts were found growing on the bottom of the ThinCert™ insert.

A variety of human epithelial cells, Keratinocytes, HaCaT cells (an immortalised keratinocyte cells line) and a human corneal epithelial cell line, were also grown in direct and indirect contact with the P(NVP-co-DEGBAC) hydrogel. At no point was a significant increase in cell viability seen when any of the cells were grown in either direct or indirect contact with the polymer. Growing keratinocytes in direct contact with the hydrogel resulted in a significant inhibition in cell viability in two out of the three experiments. However in the other experiment there was no significant difference in cell viability observed. In all three experiments where keratinocytes were grown in indirect contact with the polymer no significant difference in cell viability was observed compared to the control. HaCaT's showed similar behaviour to the keratinocytes when grown in direct contact with the P(NVP-co-DEGBAC) polymer. Two out of the three experiments showed reduced cell viability compared to the control. When HaCaT cells were grown in indirect contact with the hydrogel two out of the three experiments showed no significant difference in cell viability between the cells grown with the polymer and those grown without. One of the three experiments had significantly reduced cell viability. In all of the experiments where HCEC cells were grown in direct contact with the P(NVP-co-DEGBAC) hydrogel cell viability was significantly reduced compared to the control. Conversely in all three experiments where HCEC cells were grown in indirect contact with the polymer there was no significant difference in cell viability. Reports of PNVP based hydrogels increasing cell viability have primarily used murine 3T3 fibroblast cells (Hong, Chirila et al. 1997; Risbud, Hardikar et al. 2000). Risbud (2000) also used SiHa epithelial cells and saw a similar increase in viability when these cells were

cultured on PNVP-chitosan hydrogels. The results reported in this thesis from the growth of human dermal fibroblasts in both direct and indirect contact with the PNVP polymers confirm the results of Hong et al. (1997) and Risbud et al. (2000). However Risbud et al. (2000) also successfully cultured epithelial cells on PNVP-chitosan hydrogels. However the culture of epithelial cells on P(NVP-co-DEGBAC) hydrogels during the course of this project was unsuccessful. Devine et al. (2006) also saw an increase in the viability of hepatoma cells grown with a P(NVP-co-AA) polymer crosslinked with EGDMA..

Growing fibroblasts in indirect contact with PNVP based polymers can increase cell viability. Hong et al. (1997) suggest that when fibroblasts are grown in serum free conditions in the presence of PNVP crosslinked with diethyleneglycol dimethacrylate cell viability could be increased to approximately 90% of that seen when fibroblasts are cultured in the presence of serum. This was shown to be dependent on both the concentration of the crosslinker in the polymer and the concentration of the polymer in the culture medium. To investigate this fibroblasts were grown in both the presence and absence of serum in indirect contact with the three PNVP based polymers, P(NVP-co-EGDMA), P(NVP-co-DEGBAC) and P(NVP-co-DEGBAC-co-AA). In all cases there was no significant difference observed in the effects of the polymers on cell viability in the presence or absence of serum in the culture medium. This indicates that the effect of the PNVP hydrogels on fibroblasts is independent of the presence of serum. Risbud et al. (2000) also showed that the presence of the polymer was not always required to see an increase in cell viability. Culture medium incubated with the PNVP-chitosan polymer could also increase cell viability. This was also shown to be concentration dependent with medium containing up to 30% conditioned medium increasing cell viability.

The ability of the PNVP hydrogels to increase cell viability was unexpected, especially when the polymers were in indirect contact with the cells. Therefore a series of experiments were performed to investigate how the PNVP hydrogels affected the cell viability. A number of theories were proposed to explain how the PNVP hydrogels could affect fibroblast viability when the polymers were placed in indirect contact with the cells. 1) the polymers could be removing cell metabolic waste from the culture medium, 2) the polymers could also be removing mitogens

from the culture medium in essence forcing the cell metabolism into overdrive to compensate, 3) the polymer could be directly stimulating the cells i.e. degradation products could be released from the polymer and directly interact with the cells. Hong et al. (1997), Risbud et al. (2000) and Devine et al. (2006) all report an increase in cell viability when cells were grown with PNVP based polymers. However none of these authors were able to explain how this effect occurs. These experiments proved inconclusive with significance only obtained when washed polymers were placed into indirect contact with cells in the presence of serum. In the absence of serum it was the polymer conditioned media that produced a significant increase in cell viability. This could suggest that foetal calf serum may affect the mechanism by which PNVP can affect fibroblast viability. The direct stimulation of fibroblasts by the polymer would appear to be a promising route for further study.

At this point it was clear that a poly(vinylpyrrolidinone) hydrogel had been synthesised with material properties suitable for use as a wound dressing. The 1 wt% DEGBAC P(NVP-co-DEGBAC) hydrogel was flexible, malleable and strong. Increasing the crosslinker concentration produced harder, more brittle polymers unsuitable for use as wound dressings. Although these hydrogels proved to be non-cytotoxic, cells were unable to grow successfully on the polymers. This is not necessarily a bad property in a wound dressing as cells will not be lost when the dressing is changed. Attempts to investigate how the polymers affect fibroblast viability proved inconclusive and therefore further work would be required to determine the mechanism by which PNVP hydrogels affect cell viability when not in direct contact with the cells. The results did suggest however that the polymer may be directly interacting with the cells, perhaps via degradation products.

The second objective was to examine the cytotoxicity of the two identified anti contraction agents, β-aminopropionitrile (βAPN) and 4-methyl umbelliferone (4-MU). βAPN is a specific non-competitive lysyl oxidase inhibitor (Arem, Madden et al. 1975) and therefore inhibits collagen crosslinking. βAPN, at a concentration of 200 $\mu g.ml^{-1}$ has been shown to reduce the contraction of tissue engineered skin (Harrison, Gossiel et al. 2006c). Redden et al. (2003) showed that 1 mM βAPN significantly reduced contraction of fibroblast populated collagen gels. Nelson et al.

(1988) showed that fibroblasts treated with 0.25 and 0.5 mM βAPN had reduced migration however no differences in cell proliferation or collagen synthesis were observed, however Harrison et al. (2006c) showed that PINP production was significantly increased, indicating increased collagen synthesis, in both fibroblast and fibroblast / keratinocyte monolayer cultures treated with 200 μg.ml^{-1} βAPN. The effects of βAPN on cell viability and keratinocyte proliferation and differentiation were therefore investigated as part of this project. βAPN at concentrations of between 50-800 μg.ml^{-1} had no significant effect on the viability of fibroblast and keratinocyte mono and co-cultures. At the same concentrations βAPN had no significant effect on keratinocyte proliferation or differentiation.

4-methyl umbelliferone (4-MU) has not previously been identified as a potential anti-contraction agent in the literature. 4-MU is a specific inhibitor of hyaluronan (HA) synthesis (Rilla, Pasonen-Seppanen et al. 2004). HA affects cell adhesion, migration, proliferation and differentiation (Rilla, Siiskonen et al. 2005). Treatment of keratinocytes with 0.5 mM 4-MU has been shown to strongly, but reversibly reduce cell proliferation (Rilla, Pasonen-Seppanen et al. 2004). The effects of 4-MU on cell viability and keratinocyte proliferation and differentiation were therefore investigated as part of this project. At a concentration of greater than 1 mM 4-MU significantly reduced the viability of fibroblasts and keratinocytes in both single and co-cultures. At concentrations of 0.125 mM or greater 4-MU significantly reduced keratinocyte proliferation, at concentrations of greater than 0.5 mM 4-MU also significantly reduced keratinocyte differentiation. Ideally the effects of 4-MU on keratinocyte migration should also be evaluated.

The cytotoxic effects of 4-MU cannot be ignored. Whilst literature reports suggest that these effects are reversible when the 4-MU is removed from the cells (Rilla, Pasonen-Seppanen et al. 2004) this was not investigated as part of this project. If the treatment of the healing wound with 4-MU results in significant cell death then wound healing would be delayed. As mentioned previously when epithelialisation is delayed beyond three weeks the incidence of hypertrophic scarring increases from 33% to 78% (Papini 2004). In addition to concerns about delayed wound healing the mode of cell death would be crucial. If the cells die via necrosis and continue dying

whilst being treated with 4-MU this could lead to chronic inflammation. However, if the cells undergo a programmed cell death i.e. apoptosis then chronic inflammation would not occur. The presence of pro-inflammatory cytokines in the wound bed serve as some of the earliest signals to activate local fibroblasts and keratinocytes (Martin 1997). However prolonged inflammation delays wound healing still further and is associated with abnormal scarring (Singer and Clark 1999; Bayat, McGrouther et al. 2003). According to Eming et al. although the underlying mechanisms for the differences in the outcome of scarring is not well understood, there is substantial experimental and clinical evidence that differences in scarring reflect an altered inflammatory and/or cytokine profile between individuals or in a disease state (Eming, Krieg et al. 2007). Whilst the aim of this project is to reduce skin graft contracture it would not be beneficial to the patient to replace one type of scarring with another.

The third objective was to evaluate the efficacy of βAPN and 4-MU on contraction. The simple collagen I gel model, based on the commonly used fibroblast populated collagen gel model has been widely used since its introduction by Bell and Ivarsson in 1979 (2006) to model wound contraction. However skin and therefore burns wounds consist of more than one cell type, as well as the fibroblasts present in the dermis, keratinocytes, endothelial cells, melanocytes, neutrophils and macrophages are among the many cell types that can be found in skin and in the wound bed. Whilst fibroblast populated collagen gels are simple and convenient they are not particularly physiologically relevant. Therefore keratinocytes were introduced into the collagen gels. Oshita et al. (2006) showed that the introduction of keratinocytes into fibroblast populated collagen gels increases contraction. However even the introduction of keratinocytes into the fibroblast populated collagen gels cannot compensate for the inherent lack of dermal architecture and basement membrane. Therefore a second model based on sterilised human dermis was also used to evaluate the efficacy of the 4-MU in reducing contraction. This model has been used for many years in Professor MacNeil's group. It has been shown that in this model it is the keratinocyte that it primarily responsible for contraction (Ralston, Layton et al. 1997; Chakrabarty, Heaton et al. 2001; Harrison, Gossiel et al. 2006c).

Harrison et al. (2006c) showed that treatment of this reconstructed skin composite with βAPN at a concentration of 200 μg.ml^{-1} significantly reduced its contraction over the 28 days that the experiments were performed over. However βAPN at concentrations of between 50 – 800 μg.ml^{-1} had no effect on the contraction of collagen gels populated with both fibroblasts and keratinocytes. 1 mM βAPN had been shown to significantly reduce the contraction of fibroblast populated collagen gels by Redden et al. (2003). A 1 mM solution of βAPN is equivalent to 256.3 μg.ml^{-1}, which is slightly higher than the concentration used by Harrison et al. to reduce contraction of the reconstructed skin composite. However this concentration is within the range of concentrations examined in this project.

Previous unpublished data from the group of Dr. Mike Edwards at Glasgow University showed that contraction of fibroblast populated collagen gels was significantly reduced when these gels were treated with a 2 mM solution of 4-MU. However 4-MU does not appear to have been thoroughly investigated as a potential therapeutic anti-contraction agent. Collagen gels were therefore prepared containing either fibroblasts, keratinocytes or a 3:1 co-culture of keratinocytes : fibroblasts. These gels were treated with 2 mM 4-MU for 6 days. Unfortunately the variation in the data obtained from these experiments meant that a statistically significant reduction in contraction was only seen at days 3-5 in the keratinocyte populated gels. The fibroblast and co-culture collagen gels had no significant reduction in contraction. 4-MU successfully reduced contraction in both collagen gels and the reconstructed skin model. In collagen gels seeded with both fibroblasts and keratinocytes and treated with a concentrations of 4-MU ranging from 0 – 4 mM contraction was significantly reduced when the gels were treated with 4-MU at concentrations of 1 mM or greater. When the reconstructed skin model was treated with 4-MU at concentrations of 1 mM or greater a statistically significant reduction in contraction was seen. Analysis of the histology obtained from these composites showed that whilst the quality of the composite decreased with increasing concentration of 4-MU, the deterioration in composite quality only became significant in composites treated with 2 mM 4-MU. The only exception to this was a significant decrease in fibroblast number identified in the composite treated with 1 mM 4-MU.

At this point in the project a PNVP hydrogel had been developed with material properties suitable for use as a wound dressing. βAPN and 4-MU both appear to be promising candidates for use as anti-contraction agents. The next stage in the project was the incorporation of these drugs into the hydrogel, this proved to be challenging. Initially attempts were made to physically incorporate the βAPN in the form of 3-aminopropionitrile fumarate into the monomer blend prior to curing. This was unsuccessful, producing hydrogels with a large number of bubbles and large amounts of golden brown flecks. These were presumed to be βAPN and were found around the edges of the polymer plaque when it was removed from the oven and mould. Acrylic acid and water were added to the NVP monomer blend in an attempt to dissolve the βAPN. However this still proved unsuccessful. Therefore once the P(NVP-co-DEGBAC) and P(NVP-co-DEGBAC-co-AA) hydrogels had been washed in ethanol, discs were cut out and dried at $60^{\circ}C$ for 48 hours to produce xerogels. These xerogels were then rehydrated in PBS containing two different concentrations of the water soluble salts of the two drugs, 3-aminopropionitrile fumarate and 4-methyl umbelliferone sodium salt.

The fifth part of this project involved measuring the release of the drugs from the hydrogels. To achieve this, assays were required to measure the release of the drugs from the hydrogels. To this end an assay for the measurement and detection of proteins and amino acids the 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay was used to detect βAPN release. The TNBS assay was modified slightly to allow for the differences in reactivity between the βAPN and amino acids. Measurement of 4-MU concentration is widely used when monitoring enzymatic activity (Ritger and Peppas 1987a). In monitoring enzymatic activity a strongly fluorescent molecule, such as 4-MU, can be conjugated to the natural substrate of the enzyme. The degradation of these synthetic substrates by the enzyme releases the 4-MU giving a quantitative measurement of activity.

The exponential relation $M_t/M_\infty = kt^n$ proposed by the group of Peppas (Ritger and Peppas 1987a; Ritger and Peppas 1987b) for the release of solutes from polymeric materials was used to analyse the release of the drugs from the polymers. Where

M_t/M_∞ is the fractional release of the drug from the polymer M_t=mass of drug released at time t and M_∞ is the total mass of the drug releases by time infinity. k is the rate constant and n is the diffusional exponent. It is the value of the diffusional exponent that indicates if the drug is being released by Fickian diffusion (n=0.5) or if there is some interaction between the solute and the polymer. For cylindrical samples, such as the ones used in this study, Ritger states that for Fickian diffusion n=0.45, for anomalous (non-Fickian) transport n= 0.45<n<0.89 and for Case-II transport n=0.89 (Liu and Rimmer 2002b).

The release profiles obtained for the release of both drugs from both of the PNVP based hydrogels allowed diffusional exponents of between 0.073 and 0.232 to be calculated. These coefficients are similar to those obtained by Liu and Rimmer (2002b) for the release of 5-FU from PNVP based hydrogels. In this paper (Liu and Rimmer 2002b) the low values of these exponents was attributed to Fickian diffusion, and the interaction of the diffusate with the polymer chain. The degradation of the crosslinker also played a major role in the release of the 5-FU from the PNVP hydrogel. There appeared to be no degradation occurring in these polymers over the time frame studied and the βAPN and 4-MU were not covalently bound to the hydrogels. Therefore this last explanation does not hold for this system. Further investigation would be required to explain the mechanisms of both drug uptake and release. Unfortunately swelling studies were not performed as part of this project. These would be needed to accurately compare the loading of the drugs into the PNVP polymers to the loading of other drugs into other hydrogels.

Acrylic acid was also added to the P(NVP-co-DEGBAC) polymer to see if the release of the βAPN could be modified. The diffusional exponents obtained for the release of the βAPN from the P(NVP-co-DEGBAC-co-AA) hydrogels were as expected lower than the exponents calculated for the plain P(NVP-co-DEGBAC) hydrogel with values ranging from 0.073 to 0.171. Again these values are similar to those calculated by Liu et al. (2002b). Unfortunately at this time it has not been possible to obtain diffusional exponents for the release of other drugs from similar materials other than those quoted already. The acrylic acid had no effect on the release of the 4-MU from the hydrogels. Whilst there are slight variations in the

release of the drugs from the hydrogels the majority of the drugs are released from the hydrogels over the first 24 hours. Typically about 80% of the drug (both βAPN and 4-MU) is released over this time period. Most of the rest of the drugs were released over the following 24 hours.

The final part of this project was to examine to what extent the drug loaded hydrogels would reduce contraction in the collagen gel and reconstructed skin models. When the PNVP based hydrogels loaded with βAPN and 4-MU were placed above collagen gels seeded with both fibroblasts and keratinocytes for six days there was no significant difference in the collagen gel contraction at day 6. The P(NVP-co-DEGBAC) hydrogels themselves and the P(NVP-co-DEGBAC) hydrogels loaded from 200 and 1000 μg.ml^{-1} solutions of βAPN and 1mM solution of 4-MU also reduced contraction at 24 hours. Only the P(NVP-co-DEGBAC-co-AA) hydrogels loaded from a 1000 μg.ml^{-1} solution of βAPN and a 1 mM solution of 4-MU significantly reduced collagen gel contraction at 24 hours. However, collagen gel contraction was significantly reduced at both 24 and 48 hours when both hydrogels loaded from a 4 mM solution of 4-MU were placed in indirect contact with the gels. It was therefore decided to change the PNVP based hydrogels loaded from a 4 mM solution of 4-MU every two days over the course of the six day experiment. This resulted in a significant reduction in collagen gel contraction over the full six day period for the 4-MU loaded P(NVP-co-DEGBAC-co-AA) hydrogel. Unfortunately large error bars on day 6 for the 4-MU loaded P(NVP-co-DEGBAC) hydrogel meant that a statistically significant reduction in contraction was only seen until day 5. Time constraints meant that it was not possible to evaluate the effect of the drug loaded hydrogels on the reconstructed skin model.

9. Conclusions.

In conclusion the aim of this project was the synthesis of a hydrogel wound dressing for the prevention / reduction of skin graft contracture. Two novel poly(vinylpyrrolidinone) based hydrogels have been synthesised P(NVP-co-DEGBAC) and P(NVP-co-DEGBAC-co-AA). Both of these hydrogels have material properties suitable for use as a wound dressing.

1. Both of the novel the P(NVP-co-DEGBAC) and P(NVP-co-DEGBAC-co-AA) hydrogels are non-cytotoxic and have even shown an ability to increase the viability of dermal fibroblasts which are grown in close proximity. Generally cells will grow up to the hydrogels but not on the hydrogels. This non fouling property could be useful in reducing additional trauma to healing wound beds caused by the changing of the dressings.

2. βAPN has also been shown to have no significant toxicity associated with its use. Treatment of skin cells with βAPN at concentrations of between 50 and 800 μg.ml^{-1} had no significant effect on cell viability, keratinocyte proliferation or differentiation. However, treatment of skin cells with 4-MU showed significant toxicity associated with concentrations of 0.125 mM or greater.

3. At concentrations of greater than 1 mM 4-MU successfully reduced contraction in collagen gels populated with co-cultures of fibroblasts and keratinocytes. When collagen gels were populated with either fibroblasts or keratinocytes and treated with 2 mM 4-MU a reduction in contraction was seen but this was not significant. Despite reports in the literature that βAPN can reduce the contraction of fibroblast populated gels the βAPN had no effect on the contraction of collagen gels populated with a co-culture of fibroblasts and keratinocytes.

4. When the reconstructed skin model was treated with 4-MU at concentrations of greater than 1 mM a significant reduction in contraction was seen. This corresponded to a reduction in the quality of the reconstructed skin. At the

lowest concentration used (0.5 mM) there did not appear to be a significant reduction in quality but at the highest concentration used (2 mM) there was very little epidermis and what epidermis was visible was not attached to the dermis with no fibroblasts present in the dermis.

5. Both βAPN and 4-MU can be loaded into the PNVP based hydrogels and released in a predictable manner over 2-3 days. The addition of AA to the P(NVP-co-DEGBAC) hydrogels had very little effect on the release of the βAPN from the hydrogel.

6. PNVP hydrogels loaded with βAPN at concentrations had no effect on the contraction of collagen gels populated with a co-culture of fibroblasts and keratinocytes over 7 days. PNVP hydrogels loaded from 1 mM and 4 mM solutions also had no effect on the contraction of collagen gels populated with a co-culture of fibroblasts and keratinocytes over 7 days. However PNVP hydrogels loaded from a 4 mM solution of 4-MU significantly reduced collagen gels contraction over six days when the hydrogel was changed every two days.

These data suggest that this hydrogel could potentially be used as a wound dressing to reduce skin graft contracture in burns patients.

10. Further work.

Further work would be required to evaluate the effect of the drug loaded hydrogel on the reconstructed skin model. In addition to this further experiments would need to be carried out to look at a range loading concentrations of the drugs. Further studies looking at manipulating the drug release profiles of the βAPN could also be performed. This could involve increasing the acid content of the hydrogels either by increasing the concentration of acrylic acid in the gels or by the incorporation of other acid functional molecules such as itaconic acid which has two acid groups into the hydrogels.

A more detailed study of degradation would also be advantageous perhaps using size exclusion chromatography (SEC), high performance liquid chromatography (HPLC) or by measuring the difference in the dry weight of the polymer over a set time period. These studies could shed light on 1) if degradation products could be influencing cell viability and 2) the mode of drug release from the hydrogels.

The hydrogels produced in this thesis were produced by a thermal curing method. This resulted in hydrogels, once hydrated, that were up to 3 mm thick. This would result in a very bulky dressing. To reduce the thickness of the hydrogel it could be possible to produce the hydrogels using a UV, IR or other curing system.

Finally, to take this work to the clinic it would be necessary to conduct animal *in vivo* toxicity tests of the hydrogel, drugs and hydrogel with drugs. For this to be successful the hydrogels would also have to be able to be produced in a suitably clean environment i.e. in a suite of clean rooms.

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