Manipulating the mechanical stiffness and biological stability of collagen hydrogels

Presented by

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Abstract

Collagen is the most abundant protein in the human body. It is biocompatible, biodegradable and weakly antigenic, making it an ideal biomaterial for cell scaffolds and as the basis for many biomimetic materials. *In vitro* and *in vivo,* collagen scaffolds are in a constant state of flux, as new collagen is synthesised and existing collagen is degraded by collagenases (matrix metalloproteinases; MMP) from cells present in the scaffold.

The main aim of this work was to improve the mechanical strength and biological stability of Type I collagen hydrogels. Three approaches were investigated. Firstly, collagen hydrogels produced from collagen isolated from different species and by different isolation methods were compared to validate the method of mechanical testing. Enzymatic solubilisation was found to produce collagen hydrogels which were mechanically weaker than their acid solubilised comparators. However, enzymatic solubilisation did not affect the contraction of free-floating fibroblast populated collagen lattices.

Secondly, ACE inhibitors: captopril, enalapril and lisinopril, were added to collagen hydrogels. ACE inhibitors have long been used in the treatment of hypertension. Literature suggested ACE inhibitors may also inhibit the action of MMPs, so adding them to cell-seeded collagen hydrogels may slow degradation, thus maintaining the mechanical stiffness of the hydrogel. The effect of ACE inhibitors on hydrogels was tested over 9 days, but evidence of any change in bulk stiffness was inconclusive. Neither enalapril nor lisinopril affected cell numbers; however captopril had a

cytostatic effect. This result was reflected in contraction of free-floating hydrogels with no significant contraction of captopril hydrogels. Further testing would be required to measure mechanical properties at the cellular level but was beyond the scope of this study.

The third, and most successful approach was dehydration of hydrogels. A method of dehydrating cell-seeded hydrogels was developed, based on the plastic compression method designed by Robert Brown and colleagues (2005). A novel super-absorber, sodium polyacrylate, was used in the dehydration process, which proved extremely effective in increasing the mechanical strength of hydrogels. Gel weight decreased by 98% in 10 minutes maintaining viable fibroblast cells within the hydrogel, at a fraction of the cost of commercially available systems and without the need for any specialist equipment. After 4 weeks in culture, there was no significant difference in cell numbers in the hydrogel between hydrated and dehydrated gels, while *de novo* collagen synthesis was increased in dehydrated gels. Further, supplementing dehydrated hydrogels with ascorbic acid and hyaluronic acid promoted cell viability and collagen synthesis. This dehydration system has great potential to be further developed to 3D tissue models, and could prove invaluable to the tissue engineering community, as well as widening access to simple 3D cell culture systems.

Abbreviations

AA	Ascorbic acid
ACE	Angiotensin converting enzyme
AFM	Atomic force microscopy
ASRT	Acid soluble rat tail tendon
CFDA	Carboxyfluoro-diacetate
CHF	Congestive heart failure
CNS	Central nervous system
CS	Chondroitin sulphate
DAPI	4,6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
DPBS	Dulbecco's phosphate buffered saline
DS	Dermatan sulphate
ECM	Extracellular matrix
FACIT	Fibril associated collagens with interrupted triple helices
FBS	Foetal bovine serum
FITC	Flourescein isothiocyanate
FPCL	Fibroblast populated collagen lattice
GAG	Glycosaminoglycan
HA	Hyaluronic acid
HDF	Human dermal fibroblast
HMDS	Hexamethyldisilazane
HSD	Honest significant difference
KS	Keratan sulphate

LC-MS	Liquid chromatography-mass spectroscopy
MACIT	Membrane associated collagens with interrupted triple helices
MMP	Matrix metalloproteinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular weight
PI	Propidium iodide
PSB	Pepsin soluble bovine
PSRT	Pepsin soluble rat tail collagen
SP	Sodium polyacrylate
TIMP	Tissue inhibitor of metalloproteinase
WHO	World health Organisation
3T3	3 day transfer cell line from primary mouse embryo fibroblast

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Chapter 1

Introduction

1 Introduction

1.1 Tissue Engineering

Tissue engineering is an emerging interdisciplinary field using engineering techniques and knowledge from the life sciences to tackle problems insurmountable without such multidisciplinary collaboration (Heineken and Skalak, 1991). In this field, cells, biomaterials and chemical factors are employed to "restore, maintain or improve tissue function" (Langer and Vacanti, 1993). Biological tissues affected by infection, genetic disorders or injury can be replaced or augmented with tissue-engineered devices to this end.

An autograft, transplanting tissue from one area of the body to another within the same person; an allograft, transplanting the tissue from one person to another; or a xenograft, transplanting tissue from one species to another; can be undertaken to fulfil the need for replacement organs and tissues. However, a critical shortage of tissue and organs available for transplantation from both live and dead human donors means supply cannot meet the demand. Immune rejection is also a problem with allografts and xenografts requiring patients to have treatment with immune-suppressants for the rest of their lives, but many individuals still have problems accepting the transplanted tissue or organ. Although autografts have the distinct advantage that they will not be rejected by the recipient's immune system, suitable tissue is not always available to autograft, and the process may require a donor site leaving the patient with multiple sites of injury.

Synthetic solutions to overcome the difficulties of transplantation, based on biomaterials from either wholly synthetic or natural sources, are therefore desirable. There are two main approaches in tissue engineering:

(i) creating tissue scaffolds seeded with cells *in vitro* which are allowed to form a tissue like structure prior to transplantation (Howard et al., 2008);

(ii) creating cell scaffolds loaded with growth factors and chemical cues which can be infiltrated with native cells post-transplantation (Howard et al., 2008).

Both techniques have merits and drawbacks and can be used independently or in combination. There are a plethora of biocompatible biomaterials available from both biological and synthetic sources (Numata and Kaplan, 2011) and choosing an appropriate biomaterial for the tissue engineering application can be challenging. Synthetic materials, such as poly(caprolactone), poly(lactic acid) and poly(glycolide) (Jenkins and Little, 2019) are often easy to tune mechanically, but do not contain cell cues for cell attachment or are not easily degraded or remodelled by embedded cells. Natural ECM materials, such as hyaluronan, elastin, and collagen, when reconstituted are often more difficult to tune mechanically or may pose immunogenic problems. However, they do provide cues for cell binding and are more easily degraded and remodelled *in vivo*. Ultimately, designing scaffolds that support biologically relevant cells and allow them to differentiate, migrate and survive is important for the future of tissue engineering. Rational scaffold design must mimic the properties of native extracellular matrix (ECM) by: providing mechanical support matched to native tissues; ensuring cells can attach to the matrix; offering chemical cues to

ingrowing cells; biodegrading at a suitable rate to ensure cells replace the matrix with native ECM, and producing a minimal immunogenic response when implanted (Drury and Mooney, 2003; Brandl et al., 2007; Howard et al., 2008; Dong and Lv, 2016).

1.2 Collagen

1.2.1 Chemical Structure and function

The collagen family are the most abundant proteins in the human body. They are the main source of mechanical stability and support in the ECM (Beck and Brodsky, 1998). Collagens are genetically conserved in both invertebrates and vertebrates, being the main constituent of skin, bone, tendons, cornea, blood vessels and the ECM in general. Collagens are a family of proteins made up, at least in part, of a distinctive triple helix of three polypeptide α-chains (Rich and Crick, 1955; Kadler et al., 2007). Currently 28 different types of collagen have been described (Gelse, 2003; Bella, 2016), which can be divided into sub-families classified by structure and function (Table 1.1). All collagen types share a common structural motif: each of the α-chains form a left-handed helix with three residues per turn. The left-handed α-chains are staggered by one amino residue to form a right-handed supercoiled triple-helix (Gelse, 2003; Kadler et al., 2007). The super-coiled structure was correctly elucidated by Ramachandran and Kartha and Rich and Crick using X-ray diffraction studies (Ramachandran and Kartha, 1955; Rich and Crick, 1955). A glycine-X-Y motif is the most common triplet with proline and hydroxyproline in positions X and Y respectively (Gelse, 2003; Brodsky and Persikov, 2005; Gordon and Hahn, 2010). Post-translational hydroxylation of proline to hydroxyproline assists with inter-chain

hydrogen bonding (Bella et al., 1994). Glycine is required as every third residue as it is the smallest amino acid (Bhagavan and Ha, 2011) with a side chain of a single hydrogen atom (Figure 1.1). This allows it to fit into the central axis of the collagen triple helix thus ensuring correct folding of the collagen triple helix. Interruption of the Gly-X-Y triplet by substitution of glycine with alanine can result in diseases such as Ehlers-danlos, osteogenesis imperfecta and chondrodysplasia due to incorrect folding of the collagen molecule (Brodsky and Persikov, 2005; Broughton et al., 2006; Fratzl, 2008; Henriksen and Karsdal, 2016). The high degree of imino acids maintains the planar peptide bonding (Pauling and Corey, 1951; Beck and Brodsky, 1998; Shoulders and Raines, 2009). The chains are stabilised by interchain hydrogen bonds between C=O and N-H. A highly ordered network of hydration plays two roles: mediating hydrogen bonds between the chains within the polypeptide chain; and forming a cylinder around the chains to maintain lateral separation (Bella et al., 1994). The triple helix region is flanked by unwound telopeptide regions at each end of the molecule (Doege and Fessler, 1986); a C-propeptide, thought to initiate triple helix formation (Gelse, 2003); and an N-propeptide, thought to be involved in maintaining the diameter of the triple helix. Collagen molecules are approximately 1000 amino acids or 300 nm in length with a diameter of 13.6 Å (Bella, 2016). These molecules aggregate into collagen fibrils where molecules are stacked in parallel with a quarter stagger and periodicity of 67 nm giving a distinctive banding pattern (Chapman et al., 1990; Fratzl, 2008).

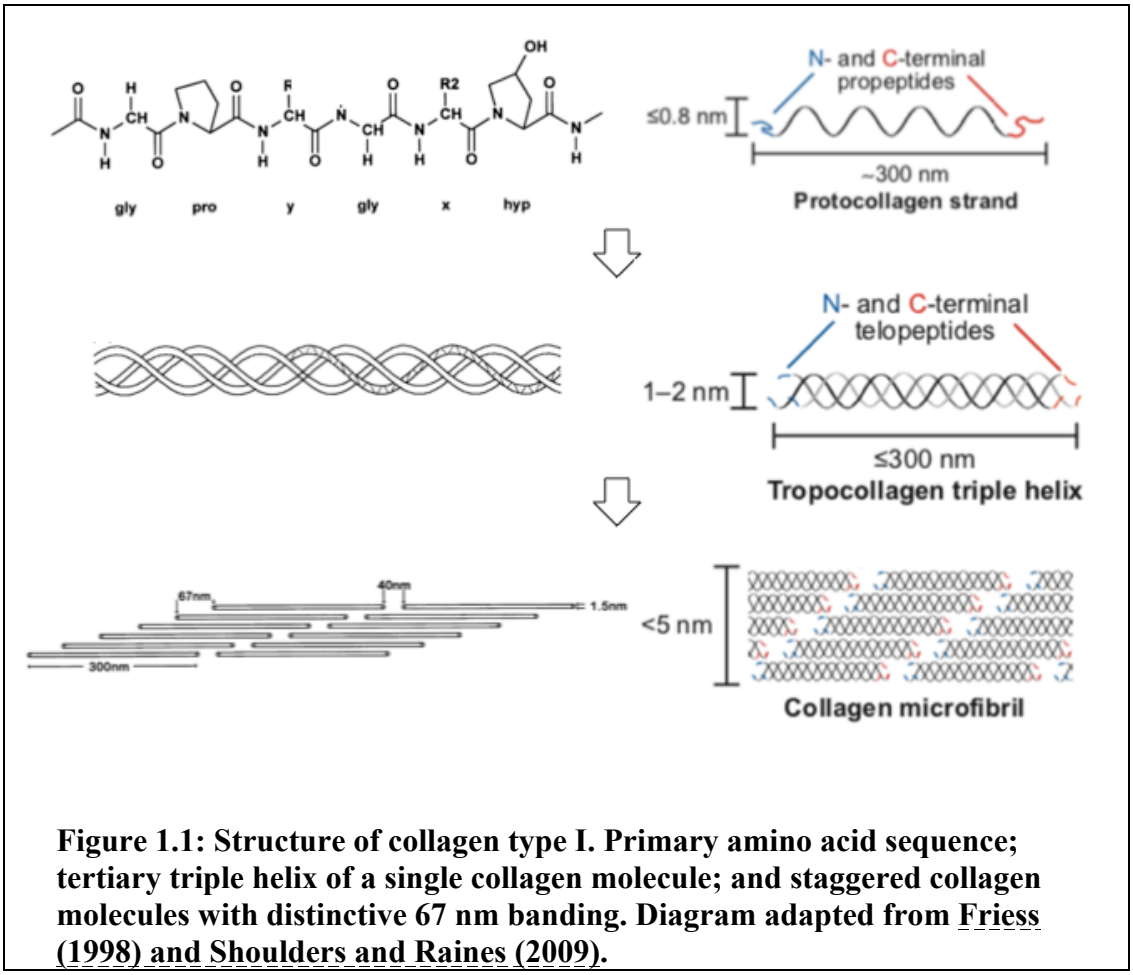


Figure 1.1: Structure of collagen type I. Primary amino acid sequence; tertiary triple helix of a single collagen molecule; and staggered collagen molecules with distinctive 67 nm banding. Diagram adapted from Friess (1998) and Shoulders and Raines (2009).

The length of the triple helix can vary dramatically between different types of collagen, from less than 10% in type XII to over 96% in type I (Ricard-Blum, 2010). The 28 different types of collagen are separated into 8 groups by their quaternary structure and function: fibril forming; basement membrane associated; microfibrillar collagens; anchoring fibrils; hexagonal network forming; fibril associated; transmembrane and multiplexin collagens (Gelse, 2003; Ricard-Blum and Ruggiero, 2005). Each collagen type is either homomeric: made up of identical polypeptide chains; or heteromeric: made up of different α chains (Brinckmann et al., 2005), each encoded by a separate gene. In total there are 45 distinct collagen genes in vertebrates (Bella, 2016).

Table 1.1 lists collagen types, quaternary structure group, distribution in the body and polypeptide chain make up.

Fibril forming collagens are the most abundant group of collagens, including types I, II, III and V. They are the main source of mechanical strength in animal tissue, and are present in tendon, skin, bone and cornea. Fibril forming collagens are produced as pro-collagens, a triple helical structure with unwound terminal pro-peptides. The C terminal pro-peptides require to be cleaved before fibrillogenesis can occur. After cleavage of the C pro-peptides, short telopeptides are exposed which contain the binding sites for fibril formation (Kadler et al., 2007). Fibrils are formed by staggering of collagen triple helices (Figure 1.1). Most fibrils are made up of different types of collagen from the fibril forming family. The molecules are cross-linked to form the fibrils with non-reversible covalent bonds resulting in strong rope-like structures (Friess, 1998). The work reported in this thesis focused solely on type I collagen, a fibril forming collagen with a perfect uninterrupted triple helix. Type I collagen is a heterotrimer of two α1 chains and an α2 chain, and it confers the bulk of the mechanical strength in tissues (Bella, 2016). Bone, skin, tendon ligaments and cornea all share type I collagen as their main component (Gelse, 2003). However, the collagen fibrils are arranged very differently in different tissues, conferring different function in each. In skin, fibrils are arranged in a basket weave, while they are arranged in organised parallel fibrils in tendons and muscle which is in line with the high mechanical stress of such tissues (Berthod et al., 2001; Svensson et al., 2017). The layout of collagen fibrils is an important factor in its function, for example in the

cornea, the layout of collagen fibres confers transparency as well as mechanical strength (Meek et al., 1987).

Table 1.1: Collagen types with descriptions of distribution and composition. Adapted from (Gelse, 2003; Myllyharju and Kivirikko, 2004; Brinckmann et al., 2005; Kadler et al., 2007; Shoulders and Raines, 2009; Gordon and Hahn, 2010). FACIT: Fibril associated collagens with interrupted triple helices. MACIT: Membrane associated collagens with interrupted triple helices. *(Gordon and Hahn, 2010), ** Types XXVI and XXVIII not easily classified (Gordon and Hahn, 2010).

Type	Classification	Distribution	Polypeptide Chains
I	Fibrillar	Bone, Skin, Ligaments, Tendon, Cornea	$\alpha1[I]_2$ α2[I]
II	Fibrillar	Cartilage, Vitreous body, Nucleus pulposus	$\alpha1[II]_3$
III	Fibrillar	Skin, Vessel wall, Reticular fibres	$\alpha1[III]_3$
IV	Network	Basement membranes	$\alpha1[IV]_2$ α2[IV] α3[IV] α4[IV] α5[IV] $\alpha5[IV]_2$ α6[IV]
V	Fibrillar	Lung, Cornea, Bone, Foetal membranes	$\alpha1[V]_3$ $\alpha1[V]_2$ α2[V] α1[V] α2[V] α3[V]
VI	Microfibrillar	Various: Skin, Cartilage, Placenta, Lungs, Vessel wall, Invertebral Disc	α1[VI] α2[VI] α3[VI] α1[VI] α2[VI] α4[VI]
VII	Anchoring fibrils	Skin, Cervix, Oral Mucosa, Dermal-epidermal junctions	$\alpha1[VII]_2$ α2[VII]
VIII	Network	Endothelial cells, Descemet's membrane	$\alpha1[VIII]_3$ $\alpha2[VIII]_3$ $\alpha1[VIII]_2$ α2[VIII]
IX	FACIT	Cartilage, Cornea, Vitreous body	α1[IX] α2[IX] α3[IX]
X	Network	Hypertrophic cartilage	$\alpha1[X]_3$
XI	Fibrillar	Cartilage, Vitreous body	α1[XI] α2[XI] α3[XI]
XII	FACIT	Perichondrium, Ligaments, Tendon	$\alpha1[XII]_3$
XIII	MACIT	Epidermis, Hair follicle, Endomysium, Intestine, Lungs, Chondrocytes, Liver	α1[XIII] Homotrimer *

XIV	FACIT	Skin, Tendon, Vessel wall, Placenta, Lungs, Liver	$\alpha1[XIV]_3$
XV	Multiplexin	Fibroblasts, Smooth muscle cells, Kidney, Pancreas	α1[XV] Homotrimer *
XVI	FACIT	Fibroblasts, Amnion, Keratinocytes	α1[XVI] Homotrimer *
XVII	MACIT	Dermal-epidermal junctions	α1[XVII] Homotrimer *
XVIII	Multiplexin	Lungs, Liver	$\alpha1[XVIII]_3$
XIX	FACIT	Human rhabdomyosarcoma	α1[XIX] Homotrimer *
XX	FACIT	Corneal epithelium, Embryonic skin, Sternal cartilage, Tendon	α1[XX] Homotrimer *
XXI	FACIT	Blood vessel wall	α1[XXI] Homotrimer *
XXII	FACIT	Tissue Junctions	α1[XXII] Homotrimer*
XXIII	MACIT	Heart, Retina, Metastatic cancer cells	α1[XXIII] Homotrimer *
XXIV	Fibrillar	Bone, Cornea	α1[XXIV] Homotrimer *
XXV	MACIT	Brain, Heart, Testis, Eye	α1[XXV] Homotrimer *
XXVI**	FACIT/ multiplexin	Testis, Ovaries	α1[XXVI] Homotrimer *
XXVII	Fibrillar	Cartilage	$\alpha1[XXVII]_3$
XXVIII**	Multiplexin	Dorsal root ganglia, Peripheral nerves	α1[XXVIII] Homotrimer *

1.2.2 Collagen as a biomaterial

The collagen family is the basis for many biomaterials currently in use in medicine today as it is biodegradable, biocompatible, non toxic and readily available (C. H. Lee et al., 2001). Pure collagen, or collagen mixed with either other materials of the natural ECM, such as gelatin or glycosaminoglycans (GAGs), or with synthetic materials, can be used to make biomimetic materials which can provide cells with binding sites for adhesion to allow repopulation of the tissues.

Collagens are only weakly immunogenic, partly due to the large number of regions of the structure that are homologous with most other mammal species. There have been few recorded cases of allergic reactions to collagen in medical devices (Lynn et al., 2004).

Collagens can be reconstituted in a variety of ways to be used for drug delivery, cell scaffolds, collagen sponges and other tissue engineering constructs.

1.2.2.1 Collagen Scaffolds

Collagen scaffolds have been used for a number of years for in vitro cell research and engineering (Elsdale and Bard, 1972). Collagen scaffolds can be prepared by a number of methods to give a variety of properties; from highly hydrated hydrogels, for soft tissue applications, to lyophilized solid collagen networks, for bone grafts and models.

The use of highly hydrated collagen hydrogels for in vivo tissue engineering applications is however, limited by their inherent mechanical weakness. Scaffolds for *in vitro* soft tissue applications are commonly based on highly hydrated collagen hydrogels. These typically contain over 99% water, making them mechanically weak and thus easily damaged. The inherent mechanical weakness of such hydrogels makes handling them difficult and limits their *in vitro* and *in vivo* applications as their mechanical weakness compared to native tissues affects the behaviour of both the scaffold and embedded cells. Their short degradation times and weak mechanical strength can also prove problematic when used as a basis for tissue regeneration.

There is, therefore, a strong imperative to develop new methods, and improve current methods, that increase the mechanical properties of collagen scaffolds, without adversely affecting the properties that make them useful for *in vivo* applications.

Fibrils within a collagen scaffold can be stiffened to better mimic in vivo tissues by various physical, enzymatic and chemical methods (Dong and Lv, 2016; Table 1.2). Physical methods such as Ultraviolet or gamma ray irradiation (Inoue et al., 2006) have been used to stiffen the collagen, as well as dehydrothermal treatment (Wang et al., 2015) which introduces crosslinks to the structure (Charulatha and Rajaram, 2003). This stiffens the collagen scaffolds without the addition of toxic chemicals to the matrix (Lee et al., 2001). Other methods for introducing crosslinks use chemicals such as glutaraldehyde or carbodiimides (Olde Damink et al., 1996; Osborne et al., 1998; Jorge-Herrero et al., 1999). Collagen structures have also been exposed to reducing sugars which react with amino acid groups to form crosslinks (Hapach et al., 2015). The main problem with chemical crosslinking methods is that the chemicals used can be toxic to embedded cells (Osborne et al., 1997). Adding the required cells to the scaffolds after crosslinking can overcome this problem, however, this limits the 3D nature of the scaffold, instead producing a 2D layer of cells on top of a collagen scaffold. Alternatively, enzymes can be used to introduce crosslinks. Two groups of enzymes are commonly used: transglutaminase and lysyl oxidase (Orban et al., 2003). These result in stable crosslink formation without involving potentially toxic chemicals. Transglutaminase catalyses crosslinking between collagen fibrils by formation of an amide bond between the γ-carboxamide and a primary amine functional group of two common collagen residues: glutamine

and lysine (Aeschlimann and Paulsson, 1994). Lysyl oxidase is known to crosslink collagen fibres to both other collagen fibres and elastin fibres (Orban et al., 2003) by covalently cross-linking lysine residues. Mechanical stability of collagen scaffolds can also be improved by making composite scaffolds. Biologically relevant polysaccharides and proteoglycans, such as GAGs, can be introduced into the scaffold improving the functionality of the scaffold for a wide range of both in vitro and in vivo applications. (Friess, 1997, Parenteau-Bareil et al., 2010, Hapach et al., 2015). A method of rapidly dehydrating collagen, called plastic compression, can also be used to improve scaffold stiffness (Brown et al., 2005). This method does not involve incorporation of toxic, non-biocompatible chemicals, and is discussed in more detail in section 1.6.1.

Table 1.2: Methods of inducing intra and intermolecular collagen crosslinks.

Method	Type	Chemistry
Dehydrothermal treatment	Physical	Removes bound water molecules forming ester and amide bonds between carboxyl and amino groups in neighbouring side chains (Lew et al., 2007; Drexler and Powell, 2011).
Ultraviolet light treatment	Physical	Bond formation between aromatic amino acids tyrosine and phenylalanine (Lew et al., 2007; Hapach et al., 2015).
Carbodiimide	Chemical	Chemicals such as EDC and NHS can form crosslinks between carboxylic acid and amino side chains of collagen molecules (Olde Damink et al., 1996; Hapach et al., 2015).
Gluteraldehyde	Chemical	Gluteraldehyde can be used to crosslink lysyl and hydroxylysyl side groups of amino acids, being incorporated in the process (Jorge-Herrero et al., 1999; Hapach et al., 2015).
Lysyl oxidase	Enzymatic	Catalyses covalent cross-linking of lysine residues (Pinnell and Martin, 1968; Piersma and Bank, 2019).
Transglutaminase	Enzymatic	Catalyses amide cross-link formation between glutamine and lysine (Orban et al., 2003).

1.2.2.2 Drug Delivery

Several collagen biomaterials are available as useful drug delivery materials. The most common collagen based drug delivery system incorporates drugs such as antibiotics into a collagen corneal shield (C. H. Lee et al., 2001). The drug is incorporated during the fabrication process, and then released slowly on application to the eye allowing prolonged contact between the drug and the cornea. The collagen shield dissolves slowly, lubricating the eye in the process. No further action is required to remove the collagen scaffold, thus reducing the risk of further damage or infection to the eye. Clinically, this is an extremely attractive prospect as it can deliver a targeted antibiotic topically, whilst also providing a protective shield to the

site of injury. There are many other clinical delivery applications both currently available and in development, such as delivery of various antibiotics, antiseptics, anti-inflammatories, anti-fungal and anti-cancer drugs (C. H. Lee et al., 2001; Albu et al., 2011). There are also several clinical applications involving collagenous hydrogels, which are useful in the treatment of bone fractures. Cytokines, such as bone morphogenetic 2 and 7 proteins (INFUSE, Medtronic; OP-1, Stryker) can be incorporated into collagen hydrogels and used as bone grafts, to promote healing in bone fractures, orthopaedic surgery procedures and oral surgery (McKay et al., 2007; Vaccaro et al., 2008). Drugs can be incorporated into collagen scaffolds either by hydrogen or covalent bonding or by entrapment. This allows controlled release of drugs with different chemistries, topically. The type of entrapment plays an important role in the rate of drug release. Simple entrapment by fibrils, in either hydrogels or lyophilised collagen networks begins to release the drug as soon as fluid diffuses into the network or as the network of fibrils begins to degrade (C. H. Lee et al., 2001). When drugs are covalently or hydrogen bonded into the network, the release profile can be significantly slower, and controlled by rate or method of degradation of the scaffold material (Li and Mooney, 2016).

1.2.2.3 Bioengineered Tissues

Collagen has been used for a number of tissue engineering applications such as wound dressings to replace skin lost to serious burns or as a result of infection such as diabetic foot ulcers (Holmes et al., 2013). The skin replacement can take the form of a collagen sponge containing dermal cells to encourage skin regrowth. Collagen sponges are useful in skin replacement as they keep the wound clean and moist, a

critical condition for treatment of burns victims. The sponge also has the advantage that it is biodegradable so can be consumed in the healing process rather than requiring replacement, at the risk of further damage to the skin. Lyophilised collagen sponge and films can also be useful as bone substitutes. The collagen biomaterial can be combined with osteoinductive proteins prior to implantation resulting in induction of bone regrowth as the collagen biomaterial is broken down. Tissue constructs such as blood vessels can also be readily fabricated from collagen. These are useful for implantation as they are weakly antigenic and can be readily cellularised as they are similar to the natural ECM. This allows cell attachment and can promote vascularisation of the transplanted tissue (C. H. Lee et al., 2001; Parenteau-Bareil et al., 2010).

1.2.3 Collagen sources and isolation methods

Collagens can be isolated in a number of ways and from various species (Parenteau-Bareil et al., 2010). There are three main methods of extraction of type I collagen from native sources: using solvents of neutral salts; or acids, such as acetic acid and hydrochloric acid; and finally using acid with enzymes such as trypsin or pepsin (Miller and Kent Rhodes, 1982). Since acid solubilised collagen was found to self-assemble into fibres and fibrils, it has been used to improve cell viability in culture, especially that of fastidious cells (Ehrmann and Gey, 1956; Cole et al., 1966; Elsdale and Bard, 1972; Forgacs et al., 2003). Most methods of isolation include inhibitors of matrix metalloproteinases (MMPs) and chelating agents in the extraction solvents to limit degradation of the collagen molecules. The method of extraction is generally dependent on the source of the collagen. Collagen with minimal crosslinking is normally acid soluble while collagen with a higher degree of crosslinks requires

pepsin solubilisation. Pepsin treatment is a popular method of enzymatic solubilisation as it removes the telopeptide regions of the collagen molecule (Glowacki and Mizuno, 2008; Zeugolis et al., 2008). This has two main effects; the telopeptide region of the collagen molecule is directly responsible for collagen's immunogenicity and for crosslinking into fibrils (Ellingsworth et al., 1986; Takaoka et al., 1991; Sato et al., 2000; Walton et al., 2010). This is useful in producing collagen for biomedical applications as it reduces the risk of an immunogenic response, however, it also produces a collagen with more monomers and less oligomers, which slows the fibrillogenesis process and can affect the stiffness of resultant matrices (Zeugolis et al., 2008; Bailey et al., 2011). Collagen is the main protein in the ECM of vertebrates, making it widely available from a variety of vertebrate sources. As collagen appeared early in evolution and is well conserved between species, collagen from different sources can be used ubiquitously with minimal immunogenicity, especially when treated with pepsin (Caliari and Burdick, 2016). Many methods for extracting Type I collagen from a variety of sources have been developed. Many tissue types, such as skin, tendon, kidney, basement membranes and bone have been used as a source of collagen from a variety of mammals, such as cow, rat, sheep, humans, pig and rabbit (Bailey et al., 2011; Bannister and Burns, 1972; Ehrmann and Gey, 1956; Cole et al., 1966; Kittiphattanabawon et al., 2010; Pacak et al., 2011; O'Sullivan et al., 2006; Olde Damink et al., 1996; Zeugolis et al., 2008; Zhang et al., 2011) and birds (Bannister and Burns, 1972). More recently, methods of isolation from non-vertebrates such as marine life have been explored to overcome concerns around religion and infection from animal sources (Kittiphattanabawon et al., 2010). The by-products of the

fishing industry have been used to isolate collagen from scales, bones and skin of shark, cod and carp to name but a few (O'Sullivan et al., 2006; Kittiphattanabawon et al., 2010; Zhang et al., 2011) which can also add value to the product. Collagen isolated from fish differs from mammalian collagen as it forms a gel and melts at a lower temperature and also has a higher viscosity than mammalian collagen (O'Sullivan et al., 2006). Commercially, the main sources of collagen are bovine dermis, Achilles tendon, bone and porcine skin (Kittiphattanabawon et al., 2010). A new source of commercial collagen is jellyfish; with a type I like collagen being released to the market in 2019 (Jellagen, 2019). For use in the laboratory, acid solubilised collagen from rat tail tendons is ever popular as it is cheap, readily available and consistent (Elsdale and Bard, 1972). Improved methods for extraction are developed regularly, such as that of Pacak and colleagues who developed a 3 hour isolation which was comparable to traditional isolation methods from lamb, rabbit and human skin and rat-tail tendons (Pacak et al., 2011).

1.2.4 Collagen Degradation

Collagen is highly resistant to many forms of degradation due to its highly cross-linked structure. However, the main source of collagen degradation are MMPs. MMPs are a family of zinc dependent endopeptidases which function to modulate and regulate the ECM as well as to release membrane bound biologically active proteins (Karagiannis and Popel, 2006). Not all MMPs are capable of collagen degradation, however, MMP-1, MMP-2, MMP-8, MMP-13 and MMP-14 readily degrade collagens (Chakrabarti and Patel, 2005; Klein and Bischoff, 2011; Nguyen, 2001). The collagenase MMP-1 proteolytically cleaves a peptide bond in type I, II and III collagen, allowing the triple-helix to be unwound, and thus susceptible to

further degradation by other MMPs (Page-McCaw et al., 2007; Klein and Bischoff, 2011). MMP-2 is a gelatinase which degrades denatured collagen (gelatin), however, it also targets collagen type I, IV and V as well as elastin and vitronectin (Chakrabarti and Patel, 2005). MMP-8 has a stronger affinity for type I collagen than MMP-I and is implicated in the degradation of the ECM during an inflammatory response (Klein and Bischoff, 2011). MMP-13 targets collagen type III while MMP-14 targets collagen type I, II and III. Each MMP has a specific role in cell migration, vascularisation, inflammation or ECM turnover (Nguyen, 2001; Karagiannis and Popel, 2006; Page-McCaw et al., 2007).

1.3 Mechanical testing of hydrogels and collagenous materials

1.3.1 Effects of mechanical properties on cells

In the seminal paper of Elsdale and Bard on collagen hydrogels for 3D culture (1972), they conjectured “Snatched from a life of obscurity and installed in contemporary glass and plastic palaces, cells are in danger of becoming Pygmalion's protégés. Housed in more traditional residences constructed of water and collagen instead of plastic or glass, do cells lead primitive, less cultured lives?” The effect of the stiffness of a matrix on cultured cells has been well documented, showing stiffness to affect proliferation, behaviour and phenotype among other things (Grinnell et al., 1999; Discher, 2005; Ghosh et al., 2007; Hadjipanayi et al., 2009).

1.3.2 Different methods for testing mechanical properties of hydrogels

Collagen hydrogels are biphasic materials consisting of a solid phase, comprising a network of collagen fibrils, and a fluid phase: water. They normally have extremely high concentrations of water, typically over 99.5%, making them difficult to mechanically characterise accurately. It is known that the mechanical properties of a tissue or cell culture environment can have significant effects on the growth of cells in the tissue. This makes the mechanical properties of collagen hydrogels an important factor in designing biomaterials for cell research or for medical devices (Brandl et al., 2007). This also means that it is important to be able to measure such properties reliably. Many different measurement methods such as compression, tension and atomic force microscopy (AFM) have been used and refined over the years.

Bulk stiffness of hydrogels has been tested in both confined and unconfined compression. A method for confined compression was developed which compressed collagen hydrogels by approximately 10% in height using a porous indenter. The results were calculated using biphasic theory, which produced very accurate measurements of both mechanical and fluid properties including stiffness, peak stress, equilibrium stress and permeability (Busby et al., 2013; Knapp et al., 1997). Biphasic theory was described by Mow and colleagues (Mow et al., 1980), who stated that initially the fluid in the structure bears the load as it is incompressible, until the load is transferred to the solid network (fibrils). Uniaxial tensile extension is also popular as scaffolds are often designed to be used in tension. A dumb-bell shaped sample of the hydrogel embedded with mesh can be attached to a tensile

testing machine by grips. Mesh is usually embedded where the grips are attached to prevent the sample breaking where the stress is highest (Anseth et al., 1996), instead the sample breaks in the middle. The sample is stretched at a constant rate and the load required is recorded, until breaking. Stress and strain values are calculated and plotted and a value for Young's Modulus shows the elasticity of the material (Roeder et al., 2002). The Young's modulus also allows tests to be compared, even if they were not carried by exactly the same method. AFM has also been used to measure forces on hydrogels at a cellular level, which are then used to calculate the stiffness of the hydrogel (Brandl et al., 2007). This is a valuable technique as it is a more accurate measure of the scaffold's mechanical properties at a cellular level, as well as being non-destructive.

1.4 Skin

1.4.1 Structure and function of healthy skin

Skin is the largest organ in the human body. The main functions of healthy skin are to provide a physical barrier to water loss, infection and hazardous substances; prevent mechanical and heat injuries; retain body heat; and confer the sense of touch. Human skin consists of 3 main layers, as seen in Figure 1.2: the epidermis, the dermis and subcutaneous fat. The epidermis consists of terminally differentiated keratinocytes in layers. The lower layer consists of epidermal stem cells, which mature and migrate to upper layers as they proliferate and keratinise. The outermost layers of the epidermis are metabolically dead and are continually sloughed off in daily life. The epidermis is not vascularised, so it relies on diffusion for all nutrients from the epidermal basement membrane, which also ensures the epidermis and

dermis do not become separated under mechanical stress. The dermis is a thick vascularised layer containing many nerve endings. Type I collagen makes up approximately 70% of the dry weight of the dermis, and confers mechanical strength to the skin. Elastin, glycosaminoglycans and polysaccharides are also present, which give the skin its elasticity and flexibility (Shimizu, 2007). The main cells of the dermal layer are fibroblasts, which synthesise new collagen, glycosaminoglycans and elastin. Histocytes, mast cells and other cells of the immune system are also present in the dermis, which deals with turnover of the material and with immune stresses. The layer of subcutaneous fat varies dramatically in thickness in different areas of the body. It provides a cushion to mechanical stress and retains water and heat in the body. The three layers work in concert to protect the internal organs from mechanical, heat and chemical stress. Nutrition is essential to healthy skin, as deficiency in certain vitamins can cause diseases like scurvy, characterised by poor collagen structure, oxidative stress and skin lesions.

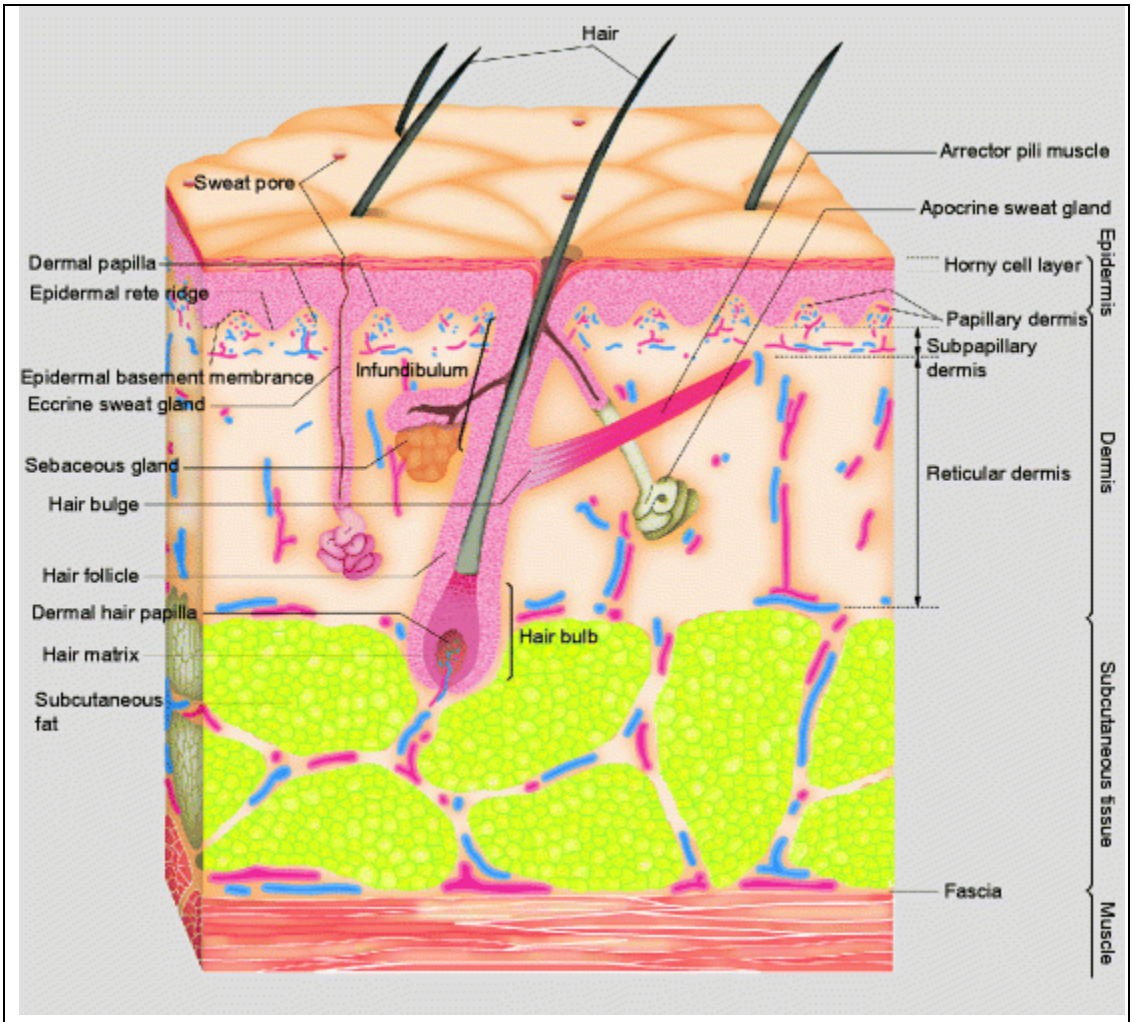


Figure 1.2: Diagram of normal skin architecture showing a layered structure and its constituent parts from (Nakagawa, 2001) via (Shimizu, 2007; Gauglitz and Schauber, 2013).

1.4.2 Burn treatment using collagen based dressings

Burns are one of the World Health Organisation's (WHO) leading causes of morbidity and cause approximately 180,000 deaths annually worldwide (World Health Organization, 2018). The gold standard for burn treatment is an autograft of partial thickness skin from another area of the patient's body (Simman and Phavixay, 2011). However, this can be problematic in burn patients with extensive or full

thickness burns, as there can be little intact tissue to autograft. An operation to remove autograft material also opens up a further site of injury where infection can be readily introduced (Smith et al., 1993). Allografts of full thickness skin primarily obtained from a donor cadaver (Burd and Chiu, 2005) or xenografts taken from other species can also be options, however, these can present problems with rejection by the immune system or transmission of disease (German, 1972; May, 1991; Yamamoto et al., 2018). This makes Type I collagen dressings and scaffolds a desirable alternative for treatment of burns victims; the high biodegradability and biocompatibility of collagen and low antigenicity make it a very attractive biomaterial for such applications. Collagen biomaterials can also be used in the treatment of ulcers, such as diabetic foot ulcers and traumatic injuries. There are collagen based skin substitutes and wound dressings (Mogoşanu and Grumezescu, 2014) currently on the market as well as other cell laden wound dressings (Pourmoussa et al., 2016). However, it is an ever expanding market and there are strong incentives to better understand ways to improve collagen based biomaterials and other potential treatments. A specific area, which is ripe for research, is improvements in the production of mechanically strong collagen hydrogels for treatment of full thickness burns and wounds, which is the ultimate objective of the work described in this thesis.

1.4.3 Available skin scaffolds and products

Since creation of an epidermal barrier by cultured autologous fibroblasts was developed in the 1980s (Gallico et al., 1984), there have been a number of epidermal, dermal and bi-layered scaffolds developed to treat full thickness burns and chronic

wounds (Auger et al., 2004; Auxenfans et al., 2009). Engineered skin scaffolds help with wound healing by providing ECM proteins, cells, chemical factors and cells to prevent moisture loss and infection (Wojtowicz et al., 2014). De-cellularised dermis tissue is one of the most popular products in clinical use (Cheema et al., 2011). It is prepared by removing cells from a complete piece of human dermis, leaving the structure and organisation of native tissue while removing the immunogenicity of an allograft. For full thickness burns and skin wounds, a de-cellularised dermal graft is an excellent material for guiding skin repair. Commercially, a number of scaffolds are available for use clinically, a selection of which are described in Table 1.3. Some of the scaffolds are sourced from lyophilised cadaveric tissue while others provide a synthetic ECM of natural or synthetic biomaterials. Cells can be expanded into the scaffolds before or after grafting and are prepared with one or more types of autologous or allogeneic cells. One of the most common novel scaffolds is Apligraf™. This is a bi-layered scaffold of bovine type I collagen populated with embedded allogeneic neonatal foreskin fibroblasts and surface seeded with keratinocytes (Pourmoussa et al., 2016). Apligraf™ has been shown to improve healing of diabetic foot ulcers and deep venous ulcers when compared to no treatment or current gold standard treatment (Edmonds and European and Australian Apligraf Diabetic Foot Ulcer Study Group, 2009; Holmes et al., 2013). Scaffolds which provide a bi-layered structure for fibroblasts and keratinocytes have been found to be much more effective in treating chronic wounds than single layer scaffolds (Wojtowicz et al., 2014), however the products available are limited. This suggests a need for development of new cell-laden bi-layered skin scaffolds.

Table 1.3: Selection of collagen based biomaterials currently available for use as scaffolds for human skin. Adapted from (Zhang et al., 2015) and (Goodarzi et al., 2018).

Scaffold name	Incorporated cells	Source of cells	Source of scaffold	Epidermis, dermis or bi-layered	Scaffold Material
Apligraf™	Neonatal foreskin fibroblasts and keratinocytes	Allogeneic	Xenogeneic	Bi-layered	Bovine type I collagen
Integra™	-	-	Xenogeneic	Dermis	Bovine type I collagen, shark chondroitin-6-sulfate, synthetic silicone epidermis
Alloderm™	-	-	Allogeneic	Dermis	Decellularised cadaveric dermis
Epicel®	Keratinocytes	Autologous	Synthetic	Epidermis	Keratinocyte sheet on petroleum jelly mesh support
Matriderm®	-	-	Xenogeneic	Dermis	Bovine type I collagen and α-elastin hydrolysate
GraftJacket	-	-	Allogeneic	Dermis	Decellularised cadaveric dermis

1.4.4 The wound healing process

Wound healing is a long and continuous process consisting of 4 distinct stages: coagulation, inflammation, migration and proliferation (Hunt et al., 2000; Broughton et al., 2006; Velnar et al., 2009). The wound healing process involves a myriad of resident and migratory cells, chemical factors and the ECM (Velnar et al., 2009). Immediately after injury, coagulation is induced by exposed collagen, and a clot of collagen, platelets, fibrin and fibronectin begins to form in the wound. This both

stops the early bleeding and provides a matrix for migrating cells to populate. Subsequently, an inflammatory response is generated which activates a complement cascade. This ultimately leads to the infiltration of neutrophils, which prevent infection of the wound, and is followed by a macrophage response, that continues the phagocytic process by releasing enzymes and chemokines. A proliferative phase then begins after the immune response has removed damaged tissue, and fibroblasts are recruited to synthesise new ECM, and replace the temporary fibrin clot. Granulation tiussue is laid down which is the basis of angiogenesis, contracture of the wound and restoration of normal structure and function of the wounded tissue (Broughton et al., 2006; Hunt et al., 2000; Velnar et al., 2009).

1.5 Chemical modification of collagen scaffolds

There are many factors that make collagen hydrogels an excellent biomaterial, however, one of the main limiting factors is that they are very mechanically weak, especially in a highly hydrated hydrogel form. Although this is useful for some soft tissue applications, strengthening the hydrogels is also an attractive prospect for a variety of other applications. Physical methods of strengthening could be useful for applications where the scaffold could be put in place whole, such as wound dressings, bone fillers, drug releasing scaffolds and corneal shields. Increasing the mechanical strength chemically could be useful for applications such as injectable fillers. Ideally, rate of degradation could be controlled to allow the scaffold to degrade as the wound healed, or to control the release of drugs over a pre-determined timeframe.

1.5.1 Inhibition of Matrix Metalloproteinases using ACE Inhibitors

Matrix metalloproteinases (MMPs) are a group of zinc dependent enzymes responsible for the degradation of the ECM and release of membrane bound proteins (Nagase et al., 2006; Klein and Bischoff, 2011). MMPs are instrumental in tissue remodelling, wound healing, angiogenesis, release of membrane bound proteins and wound healing (Jacobsen et al., 2010). It has been suggested that MMPs play a much wider role than ECM degradation, and could be novel drug targets for a variety of diseases, such as cancer and fibrotic diseases (Butler and Overall, 2009; Passos-Silva et al., 2015; Craig et al., 2015). There are 24 MMP genes present in humans which produce 23 different MMPs (Table 4.1), each with a different purpose or location (Nagase et al., 2006). MMPs can act on a range of substrates, mainly in the ECM and are inhibited by tissue inhibitors of metalloproteinases (TIMPs). In healthy tissues, MMPs and TIMPs are in an equilibrium so ECM turnover is controlled. Loss of control of MMP activity by TIMPs can lead to tissue damage and inflammation. MMP-1 or collagenase-I degrades fibrillar collagens by proteolytic cleavage of collagen molecules, leading to the unwinding of the collagen strands (Klein and Bischoff, 2011). This leaves unwound strands vulnerable to degradation. There have been several attempts to create broad spectrum MMP inhibitors, mainly based on chelating zinc ions to inhibit binding of zinc ions, and thus inhibit activation of the MMPs. These have, however failed, due to limited efficacy and a broad range of side effects caused by inhibition of the MMP family (Winer et al., 2018; Fields, 2019). Clinical trials suggest alternative methods of MMP inhibition would be more effective for *in vitro* applications than broad spectrum MMP inhibitors.

The angiotensin converting enzyme (ACE) is also a zinc metalloproteinase (Turner, 2003) . ACE inhibitors have been widely found to have an inhibitory effect on MMPs (Sorbi et al., 1993; Ihm et al., 1994; Sakata et al., 2004; Brower et al., 2007). Sorbi and colleagues (1993) showed by zymography that both captopril and lisinopril inhibited collagenases (MMP-1, MMP-8, MMP-13, MMP-18) while Sakata and colleagues found enalapril to attenuate MMP-2 and 9 (2004). MMP inhibition could be reversed by an increase in zinc concentration, suggesting a mode of competitive inhibition was involved in the zinc activation of the enzymes (Sorbi et al., 1993). ACE inhibitors could be involved in chelating zinc ions, in turn preventing activation of MMPs.

ACE plays an important role in the renin-angiotensin system, leading to increased blood pressure through a combination of vasoconstriction, increase in blood volume, and degradation of the vasodilator bradykinin. Because of this, blocking the Renin-Angiotensin system with a group of drugs called angiotensin converting enzyme inhibitors, is a traditional method of treatment for hypertension (Robertson, 1994; Passos-Silva et al., 2015). ACE inhibitors are also widely used in the treatment of congestive heart failure (CHF) and during recovery from myocardial infarction (Robertson, 1994; Pfeffer, 1998; Packer et al., 1986). Their mechanism of action to reduce hypertension is to block the conversion of Angiotensin I to Angiotensin II, in turn lowering arteriole pressure and increasing natriuresis (Herman and Bhimji, 2018). There are a number of orally available ACE inhibitors which can be divided into three groups, based on their chemical structure. ACE inhibitors either contain a sulfhydryl, dicarboxylic or phosphorous group (Herman and Bhimji, 2018). ACE is

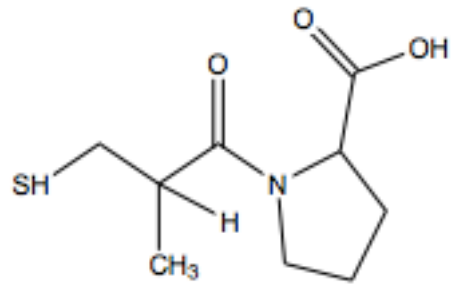
also known to inactivate bradykinin, a potent vasodilator, by cleavage of its peptide bond (Erdös, 1977).

Three ACE inhibitors were used in this work. Captopril was the first ACE inhibitor available on the market, approved by the FDA in 1981, and the only ACE inhibitor that contains a sulfhydryl group. This was successful in the treatment of most types of hypertension (Duchin et al., 1982). However, captopril had significant side effects, most commonly a non-treatable cough and metallic taste. This was thought to be caused by the sulfhydryl group contained in its structure (Packer et al., 1986). Subsequently, ACE inhibitors were found to have similar efficacy as diuretics, calcium channel blockers, β blockers and methyldopa for the treatment of hypertension (Robertson, 1994; Hansson et al., 1999). Enalapril was developed to combat the side effects of captopril by replacing the sulfhydryl group with a dicarboxylic group. It is a prodrug, an ester of enalaprilat and ethanol, to improve oral bioavailability (MacFadyen et al., 1993). It is now on the WHO list of essential medicines, as one of the most commonly prescribed and effective drugs to treat hypertension and CHF (World Health Organization, 2019). Lisinopril is a lysine analogue of enalaprillat, giving it a very similar structure (Beermann, 1988). This adaptation means lisinopril is orally active, rather than a pro-drug.

Claridge and colleagues (Claridge et al., 2004), found treatment with ACE inhibitors reduced the incidence of strokes and myocardial infarction independently from reduced hypertension. As strokes and myocardial infarction are both caused by the rupture of a fibrotic cap or plaque, Claridge and colleagues conjectured that ACE

inhibitor treatment increased the stability of the plaque by increasing synthesis of Type III collagen (Claridge et al., 2004). Inhibition of MMPs has also been suggested as a method in the treatment of CHF with ACE inhibitors. CHF is characterised by the dilation of the left ventricle (Brower et al., 2007). Patients with CHF also have increased levels of MMPs (McElmurray et al., 1999). It is thought that , in both CHF and post-myocardial infarction, ACE inhibitors reduce the remodelling of the smooth muscle and cardiomyocytes by MMPs (Herman and Bhimji, 2018) in turn reducing the loss of structure and function of the cardiac muscle (Brower et al., 2007).

(a)



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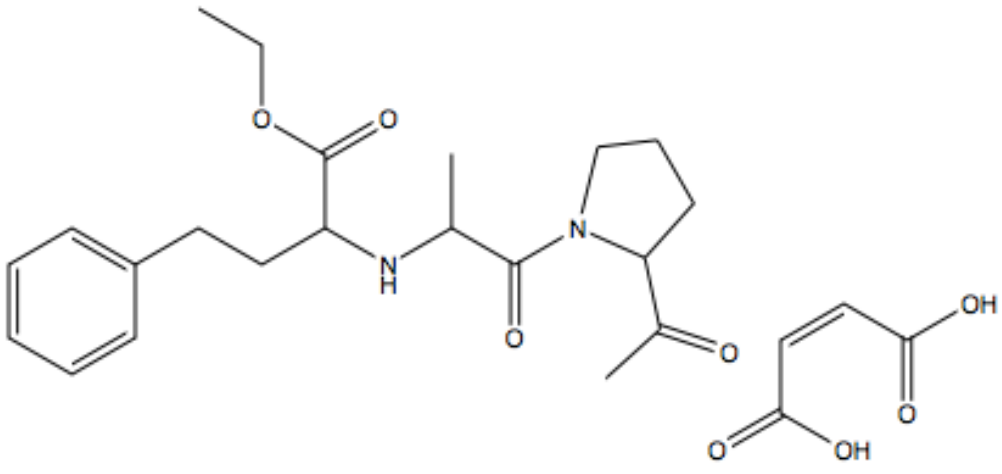

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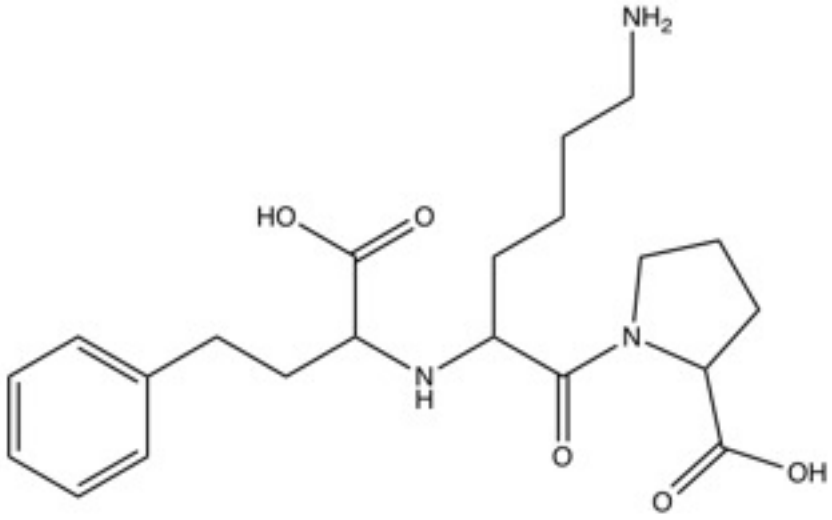


Figure 1.3: Chemical structure of ACE inhibitors: (a) Captopril; (b) Enalapril Maleate; (c) Lisinopril. Captopril was the first, and smallest ACE inhibitor discovered, and is the only ACE inhibitor which contains a sulfhydryl group. Enalapril maleate is a prodrug, an ester of the active drug enalaprilat and ethanol, to create an orally available drug. Lisinopril is the lysine analogue of enalapril, which makes it an orally active drug. It is one of the largest ACE inhibitors.

1.5.2 Effect of Glycosaminoglycans on collagen hydrogels

Glycosaminoglycans (GAGs) or mucopolysaccharides are an important family of highly sulphated long unbranched polysaccharides present in the extracellular matrix (ECM). There are 4 groups of GAGs: heparin/heparan sulphate; chondroitin sulphate/dermatan sulphate; keratan sulphate; and hyaluronic acid. Apart from keratan sulphate, all GAGs are made up of repeating units of an uronic acid (either glucuronic acid or iduronic acid) and an amino sugar (either N-Acetylglucosamine or N-Acetylgalactosamine). Unlike proteins or nucleic acids, GAGs are very

heterogeneous because their production is not ‘template driven’ (Scott and Panitch, 2013), instead it is dynamically controlled by the processing enzymes present (Raman et al., 2005). GAGs are highly polar which allows them to attract water while also being very viscose and relatively incompressible.

1.5.2.1 Heparan sulphate

Heparan sulphate is a sulphated glycosaminoglycan disaccharide polymer attached to a protein core, most of its disaccharide units are glucuronic acid linked to *N*-acetylglucosamine (Lindahl et al., 2015). It is produced in the Golgi apparatus and is expressed on the cell surface, in the extracellular matrix and in basement membranes (Sarrazin et al., 2011). Heparan sulphate plays important roles in ligand binding and cell signalling, mainly in immunity and inflammation, and in angiogenesis. In the immune system, Heparan sulphate is known to interact with soluble signalling factors and toll like receptors to control cell adhesion, cytokine and chemokine signalling and mediation of the immune response (Davis et al., 2013). Heparan sulphate is also essential for initiating angiogenesis (Zhou et al., 2004). It has been widely used in tissue engineering applications, especially in inducing angiogenesis in tissue scaffolds (Pieper et al., 2002; Brouwer et al., 2013).

1.5.2.2 Chondroitin sulphate and dermatan sulphate

Chondroitin sulphate (CS) and dermatan sulphate (DS) are GAGs which are closely linked in structure and can exist as co-polymers. CS is a long polymeric chain of repeating disaccharide units, consisting of N-acetylgalactosamine and glucuronic

acid (Silbert and Sugumaran, 2002), and is produced in the Golgi apparatus. Glucuronic acid molecules undergo epimerisation to iduronic acid to produce DS from CS. Like heparin sulphate, CS/DS are connected to a protein core and displayed on the cell surface of nearly all cells, in basement membranes and in the ECM (Jones et al., 2010; Li et al., 2013; Izumikawa et al., 2014). CS/DS play important roles in development of the CNS, signalling, wound repair, signalling infection, cell division and as structural components (Trowbridge and Gallo, 2002; Sugahara et al., 2003). The chain length and thus molecular weight of CS/DS varies widely from 5-70 kDa, as does the extent of sulfation of the molecule (Köwitsch et al., 2018). This accounts for some of the variation in function. As part of the proteoglycans decorin and biglycan, CS/DS helps to regulate the fibrillogenesis of collagen and protect against enzyme degradation (Köwitsch et al., 2018; Kadler et al., 2007). Many CS/DS containing proteoglycans have structural function including entrapping water and maintaining osmotic pressure as well as providing viscoelasticity to tissues (Iozzo and Schaefer, 2015). The mix of structural function and their function as signalling molecules, give CS/DS many desirable attributes for tissue engineering of skin scaffolds.

1.5.2.3 Keratan sulphate

Keratan sulphate (KS) is mainly found in the cornea although it can also be found in other tissues. It is a polymer of repeating units of N-acetylglucosamine and galactose disaccharide. Like CS/DS and HS, KS is produced in the Golgi apparatus and is attached to a core protein. There are 3 types of keratan sulphate which all have different purposes in different tissues: KS I, KS II and KS III. The three differ in

their level of sulfation and the oligosaccharides which link them to their core protein (Funderburgh, 2002). KS I is the N-linked corneal KS responsible for maintaining hydration and transparency in the cornea (Köwitsch et al., 2018). KS II is only linked to aggrecan, the main proteoglycan of cartilage (Funderburgh, 2002). KS III is normally found linked to proteins and in proteoglycans in the brain and CNS (Funderburgh, 2000, 2002).

1.5.2.4 Hyaluronic acid

Hyaluronic acid or hyaluronan (HA) is a simple polysaccharide and the only non-sulphated GAG. It is synthesised by hyaluronan synthases, which are transmembrane proteins embedded in the cellular membrane (Laurent and Fraser, 1986; Litwiniuk et al., 2016), and transported to the extracellular matrix (Weigel and DeAngelis, 2007; Köwitsch et al., 2018). HA is made up of alternating β-D-glucuronate and *N*-acetyl-β-D-glucosamine sugars (Litwiniuk et al., 2016) and has a very large molecular weight (MW) normally ranging from 1-8 MDa (Cowman et al., 2015); although it can also be synthesised as a low MW polymer. High and low MW HA have different effects: high MW HA has immunosuppressive and anti-inflammatory properties while low MW HA initiates a pro-inflammatory response (Rayahin et al., 2015; Litwiniuk et al., 2016; Köwitsch et al., 2018). Generally in healthy skin, the molecular weight of HA is in the region of 4-6 kDa although low MW HA can be found in healing wounds (Cowman et al., 2015). The extensive hydrogen bonding of HA gives it a flexible ‘worm-like’ structure (Hart and Copeland, 2010), which allows the molecule to fill gaps in the extracellular matrix.

After collagen, HA is one of the most abundant molecules in the extracellular matrix. Human skin contains about 0.4-0.5 mg/g HA (wet weight), mostly in the dermis (Anderegg et al., 2014; Lee et al., 2016). Hyaluronic acid has a fast turnover rate, with a half-life of only 1 day (Fraser and Laurent, 1989; Lee et al., 2016). It is constantly degraded by reactive oxygen and nitrogen species, hyaluronidases and MMPs meaning the ECM is constantly remodelling itself. The polar nature of the hyaluronic acid molecule gives a very hydrophilic molecule which can absorb up to one thousand times its own volume in water (Khunmanee et al., 2017). HA has important roles in lubricating and cushioning the skin (Goa and Benfield, 1994) and maintaining hydration in the skin (Juhlin, 1997; Papakonstantinou et al., 2012). It also has important roles in cell proliferation and migration although these are not fully understood.

The main receptor for HA is CD44 which is present on the surface of nearly all human cells (Misra et al., 2015). The interaction of CD44 and HA is an essential part of the wound healing process as it signals fibroblasts from surrounding tissues to migrate into the wound and activate the wound healing process (Litwiniuk et al., 2016). Over the years, HA has been used extensively in dermal fillers (Gold, 2007; Anderegg et al., 2014) and tissue scaffolds (Liu et al., 2004; Shah et al., 2008; Numata and Kaplan, 2011; Wang et al., 2013) as it promotes skin hydration and cell viability in the scaffold. It is an excellent molecule for tissue engineering applications because of its physicochemical properties as well as being biocompatible, biodegradable and non-antigenic.

1.5.3 Ascorbic Acid

Ascorbic acid (AA), more commonly known as Vitamin C is an essential nutrient gained exclusively from the diet, as it cannot be synthesised by humans (Carr and Frei, 1999). A deficiency of AA results in scurvy, a disease which causes poor wound healing, bleeding gums, weak skin and fatigue and can lead to death if untreated (Boyce et al., 2002; Li and Schellhorn, 2007). Ascorbic acid plays a critical role in collagen synthesis and as an antioxidant, making it essential in skin health (Li and Schellhorn, 2007; Tajima and Pinnell, 1996; Pullar et al., 2017). In the collagen synthesis pathway, AA acts as a co-factor in the proly and lysyl hydroxylase enzymes which catalyse the hydroxylation of proline and lysine (Rebouche, 1991; Carr and Frei, 1999). Hydroxyproline is essential in crosslinking the collagen triple helix. Collagen synthesised in AA deficient conditions is less stable as proline is not hydroxylated, thus does not form collagen crosslinks (Tajima and Pinnell, 1996). Ascorbic acid has been shown to promote collagen gene expression (Pullar et al., 2017), stimulating Type I and III collagen synthesis (Tajima and Pinnell, 1996). Research has shown AA increases proliferation and migration of both keratinocytes and fibroblasts *in vitro* and *in vivo* (Boyce et al., 2002; Pullar et al., 2017). Ascorbic acid is a potent antioxidant (Boyce et al., 2002). This helps combat oxidative stress in skin and other tissues, and this has been exploited to treat cancer and cardiovascular disease (Li and Schellhorn, 2007).

1.6 Dehydration of collagen hydrogels

1.6.1 Plastic compression

Plastic compression was developed by a group of researchers at University College London led by Professor Robert Brown. The technique sought to overcome the inherent mechanical weakness of collagen hydrogels. Naturally, collagen hydrogels prepared with cells strengthen over time as cells remodel the matrix. A method to accelerate this natural process was developed, without the need for remodelling of the scaffold by resident fibroblasts (Brown et al., 2005). Methods were crude but very effective: the collagen hydrogel was removed from a casting chamber then placed on absorbent paper and covered with a non-porous surface such as a glass slide with weights added to the surface. The method used gravity to force water out of the hydrated collagen network into the absorbent paper below which dramatically increased the mechanical stiffness of the scaffold and produced a tissue-like structure. The method was developed to increase the dehydration of the scaffolds further by adding weights of 10 or 60 g on top of the gels in the dehydration apparatus (Abou Neel et al., 2006). Scaffolds were then rolled after the first dehydration step to increase the dehydration and thus collagen concentration (Figure 5.1). This produced multi-layered tissue-like structures with improved mechanical responses (Abou Neel et al., 2006), however they did not support viable cells (Bitar et al., 2007). There has been much research on plastically compressed gels to improve the method further (Alekseeva et al., 2011, 2012) and increase cell viability within plastically compressed scaffolds (Cheema and Brown, 2013; Ardakani et al., 2014). Plastic compression has also been commercialised and produced by Lonza under the name RAFT™ (Lonza, 2019). This is an all in one system which can

produce a plastically compressed acid soluble rat-tail collagen scaffold containing viable cells in a 24 or 96 well plate, however it is expensive, costing over £100 (at time of writing) for a single 24 well plate.

1.6.2 Super-absorbers

Super-absorbers are extremely hydrophilic polymers which can absorb large amounts of water, comparative to their weight, forming a rigid gel. Super-absorbers are useful for a variety of applications such as cleaning e.g. spill kits; personal hygiene products e.g. infant nappies; food e.g. preventing moisture from solidifying sugar in transport; and in agriculture e.g. maintaining soil moisture. Super-absorbers are normally cross-linked networks of polymer containing ionic groups that create a difference in osmotic pressure. On wetting, the super-absorber draws liquid into the network, subsequently trapping the liquid phase in stable hydrogel. (Liu and Guo, 2001). There are numerous super-absorbers available, depending on the requirements of the application. These are discussed further in Chapter 5.

1.7 Aims and Hypotheses of this thesis

The main research theme running throughout this work was to improve the mechanical properties and increase the biological stability of collagen hydrogels, primarily for dermal cell scaffold applications. Throughout the project, the method changed by which this objective was achieved, however the aim to improve the control over stiffness and biological stability remained the same.

(i) Differences in fibrillogenesis, bulk stiffness and behaviour of collagen hydrogels containing cells were investigated to compare collagen isolated from different sources and by different methods. The diversity of collagen sources and preparation methods means there is little literature to compare how the preparations differ from one another. It is therefore difficult to translate how research completed on one preparation can be translated to collagen isolated from other species using different isolation methods. The first aim of this thesis was to compare how acid and pepsin preparations from different species and tissues differed, and therefore how translatable research on laboratory preparations translated to commercial preparations, which may be used in patients.

(ii) Another aim of this thesis was to validate a method of mechanical testing, adapted from Busby and colleagues (2013), to accurately measure small changes in stiffness. Testing the stiffness of hydrogels of different concentrations of collagen from the same preparation was used as a method to validate the testing process.

(iii) Previous work by Busby and colleagues suggested that ACE inhibitors could increase the bulk stiffness of hydrogels (Busby, 2013), and improve cell viability without increasing the rate of collagen degradation. Methods to increase the mechanical strength and stability of collagen gels were further investigated and the range of ACE inhibitors expanded to include captopril, enalapril and lisinopril. The selected ACE inhibitors were incorporated into

collagen hydrogels to study whether these drugs affected the stiffness or mechanical stability of collagen hydrogels. Collagen hydrogels were prepared with and without fibroblasts to measure the impact of ACE inhibitors on the cell population. Three hypotheses were proposed for the mechanism of action of the ACE inhibitors on the hydrogels: a physical scaffolding effect, a biological inhibition or a chemical interaction. Two further molecules, collagenase inhibitor and acetylcysteine, were used to mimic elements of each drug to investigate the mechanism of action.

(iv) An alternative method to quickly and cost effectively improve the stiffness of collagen hydrogels was investigated. There has been substantial research published on rapid fabrication of plastically compressed collagen hydrogels (Brown et al., 2005). This is an excellent method to quickly increase the stiffness of collagen hydrogels, which does not require inclusion of toxic and non-biocompatible materials. However, it often requires expensive absorbents, specialist equipment or does not support cells embedded in the scaffold. The final aim was to develop a method of plastic compression that could be completed in any laboratory equipped for cell culture, would reduce the cost of the technique and support viable cells within the structure. This aim was extended to augment the dehydrated scaffolds with micronutrients and GAGs to produce a more tissue-like material which mimicked native skin. Collagen hydrogels were prepared with human dermal fibroblasts, then dehydrated with the super-absorber, sodium polyacrylate. Ascorbic acid and hyaluronic acid were added to

the hydrogels, and the cell viability, *de novo* collagen synthesis and cell morphology were measured to assess their viability as cell scaffolds.

The investigations carried out to achieve these are detailed as follows:

- Chapter 3: Comparison of collagen from different sources
- Chapter 4: Manipulating collagen properties with ACE inhibitors
- Chapter 5: Controlled dehydration of collagen hydrogels
- Chapter 6: Summary of Results and Further Work

Chapter 2

Materials and Methods

2 Materials and Methods

2.1 Materials

All chemicals used were of analytical grade and unless otherwise stated were purchased from Sigma Aldrich, UK. Rat tails for the preparation of both acid soluble and pepsin soluble rat tail (ASRT and PSRT) collagen were kindly donated by Strathclyde University Biological Procedures Unit from control animals used in procedures taking place in research carried out in Strathclyde University. Pepsin soluble bovine (PSB) collagen was kindly donated by Collagen Solutions Plc., Glasgow.

2.2 Cell Culture

2.2.1 Cell types used

Two cell types were used to study their effect on collagen scaffolds: an immortalised mouse fibroblast cell line (3T3; Catalogue number ATCC CRL-1658, ATCC.) and primary Human Dermal Fibroblast cells (HDF; Catalogue number: 106-05A, Sigma Aldrich).

2.2.2 Maintaining a cell line

Both the 3T3 and HDF cells were maintained in complete Dulbecco's modified Eagle's Medium (DMEM; Catalogue number: BE12-604Q, Lonza) supplemented with 10% foetal bovine serum (FBS; Biosera, Labtech); 50 units/ml penicillin (Sigma Aldrich); 50 μg/ml streptomycin (Sigma Aldrich); and 1% (v/v) non-essential amino acids (Lonza). Cells were cultured in 75 cm^2 Nunclon delta coated cell culture flasks (Thermofisher Scientific) in an incubator (37°C with 5% CO_2). Versene was

prepared by dissolving: 12 g of sodium chloride; 0.3 g of potassium chloride; 1.73 g of anhydrous disodium hydrogen phosphate; 0.3 g potassium dihydrogen phosphate; 0.3 g ethylenediaminetetraacetic acid; and 1 % phenol red in a 1.5 l solution with deionised water.

3T3 cells were passaged every 3-4 days at a split ratio of 1:5 or 1:10. To passage, the medium was discarded and the cell monolayer rinsed twice with versene. Sufficient trypsin in versene (1:3) to cover the monolayer was added and the flask agitated by gently tapping the side of the flask. After the monolayer was detached, a small volume of complete DMEM was added to inhibit the action of trypsin and pipetted up and down to break up detached sheets of cells. The cells were pipetted into a new cell culture flask to give the desired split ratio. Freshly prepared complete DMEM, warmed to 37 °C, was added to give a total volume of 20 ml in a 75 cm^2 flask.

HDF cells were maintained as above. The medium was replaced every second day with freshly prepared complete DMEM at a ratio of 1 ml medium to 5 cm^2 of the flasks' surface area. When confluent, the cell layer was rinsed twice with versene then trypsinised to detach the cell layer. Once detached, 10 ml of media were added to inhibit the action of trypsin. The suspension was transferred to a sterile conical centrifuge tube and centrifuged at 220 x g for 5 minutes to pellet the cells. Cells were re-suspended in 5 ml of DMEM and counted with a haemocytometer. A density of 3000 cells/cm^2 was seeded in a new cell culture flask and media added to maintain the 1 ml: 5 cm^2 ratio.

2.2.3 Seeding Cells

For the experiments investigating the effects of incorporating cells into hydrogels, cells were treated with trypsin as in 2.2.2. Instead of splitting the cells into a fresh flask, cells were counted using a haemocytometer and microscope to estimate the number of cells/ml. The required volume of cells was then transferred to a sterile conical centrifuge tube and centrifuged at 800 rpm for 10 minutes. The cell pellet was re-suspended in 1 ml of media and seeded at the desired cell density/cm^2 onto tissue culture plates, on the surface of set collagen hydrogels or within collagen hydrogels prior to setting.

2.3 Preparation of type I collagen

2.3.1 Acid and pepsin extraction of type I collagen from rat tail tendons

Preparation of ASRT and PSRT collagen is outlined in Figure 2.1. ASRT collagen was prepared by a method adapted from Elsdale and Bard (1972). Briefly, rat-tail tendons were extracted, dissolved in 0.5 M acetic acid for 48 hours, strained and the dissolved fraction dialysed against 10 % Dulbecco's Modified Eagles Medium (DMEM) twice for 24 hours each. The product was sterilised by centrifuging at 10,000 RPM for 2 hours at 4 °C. Collagen concentration was determined by desiccating 3 ml of the final product on a glass petri dish in a 37 °C oven then weighing.

PSRT collagen was prepared by a method adapted from Zeugolis (2007). The un-dissolved fraction, strained from the acid soluble preparation, was dissolved in 0.5 M acetic acid containing 0.5% (w/w) porcine pepsin (Sigma Aldrich, UK) for 48 hours.

The pepsin was neutralised by raising the pH of the collagen solution to pH 9 for 1 hour with sodium hydroxide, before returning to pH 2 with hydrochloric acid. The PSRT collagen was dialysed, centrifuged and collagen concentration determined as for the ASRT collagen.

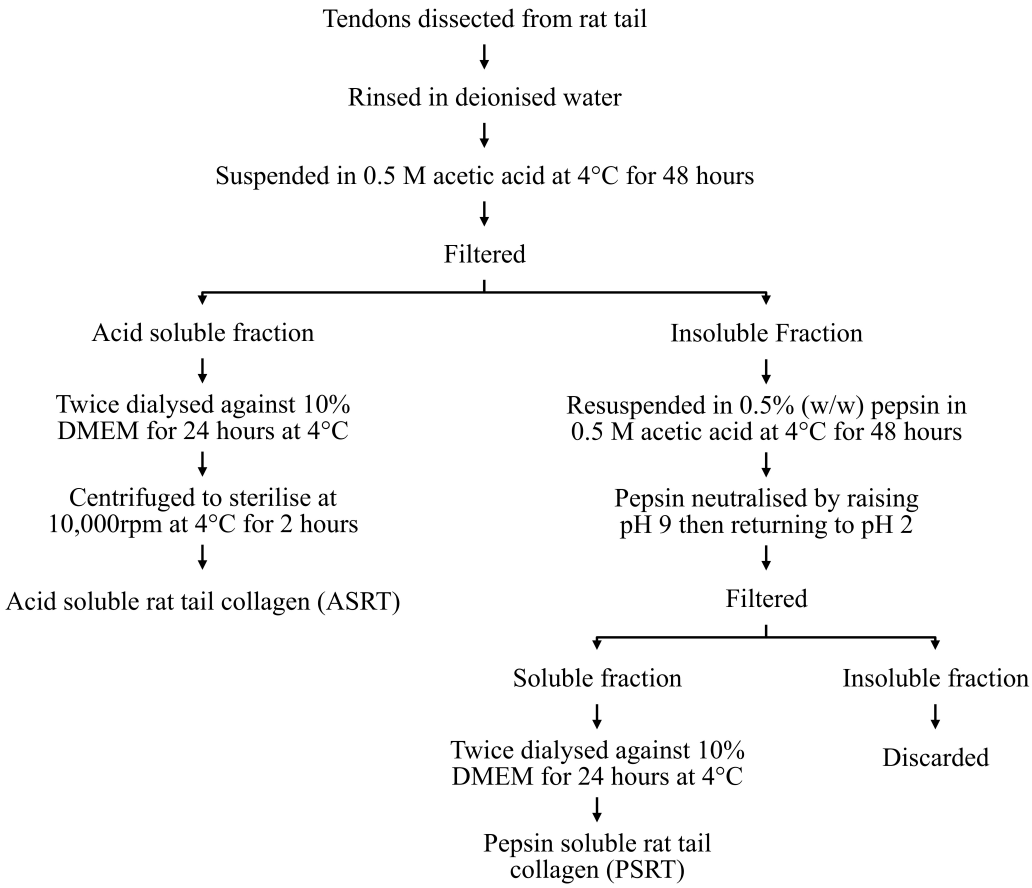


Figure 2.1: Overview of preparation method of ASRT and PSRT collagen. Preparation method for ASRT collagen adapted from Elsdale and Bard (1972) and method for PSRT collagen adapted from Zeugolis et al. (2007).

2.3.2 Preparation of collagen hydrogels

ASRT, PSRT and PSB collagen hydrogels were all prepared in the same way as follows, as indicated in Figure 2.1. The relevant collagen solution was mixed with a 2:1 solution of 10 x DMEM: 0.4 M NaOH and 1/1000 acetic acid (for PSB, 0.01 M

HCl) to give the desired final concentration of collagen in the hydrogel. Example volumes are displayed in Table 2.1. It should be noted that 2:1 DMEM: NaOH was always added at a concentration of 10% of the final volume of the collagen solution. The three components were mixed on ice in a sterilised glass beaker. To set the collagen hydrogel, 1 M NaOH was added drop-wise, whilst mixing, to raise the pH of the solution to 8-8.5, until a bright pink colour was achieved. The desired volume of collagen gel solution was pipetted into tissue culture plates or casting plates, then set in an incubator (37°C with 5% CO_2) for 2 hours, unless otherwise stated. After setting, freshly prepared complete DMEM was added to fully polymerise each gel and they were maintained in the incubator until required.

Table 2.1: An example of the volumes of component solutions in a collagen hydrogel

	Collagen solution (e.g 5.4 mg/ml)	1/1000 acetic acid (0.01 M HCL for PSB)	2:1 10x DMEM: 0.4 M NaOH
10 ml 0.2% collagen	20/5.4 = 3.7 ml	5.3 ml	1 ml
10 ml 0.3% collagen	30/5.4 = 5.6 ml	3.4 ml	1 ml
10 ml 0.4% collagen	40/5.4. = 7.4 ml	2.6 ml	1 ml

2.3.3 Preparation of collagen hydrogels with cells

Cells were prepared as in section 2.2.3 for inclusion in collagen hydrogels. The number of cells per square centimetre of surface area of the tissue culture plate was calculated. The pH of the collagen solution was raised to 8-8.5 as in section 2.3.2, to initiate setting of the solution, prior to adding cells and mixing. A final solution of collagen containing cells was pipetted into tissue culture plates or casting plates and set in an incubator (37 °C with 5% CO_2) for 2 hours, unless otherwise stated.

2.3.4 Preparation of collagen hydrogels containing ACE inhibitors

The ACE inhibitors used in this work, Captopril, Enalapril maleate and Lisinopril (Sigma Aldrich, UK), were all dissolved in the hydrogels at a concentration of 1 mM. To incorporate the drugs into the hydrogel, the mass of ACE inhibitor required to give a final concentration of 1 mM in the hydrogel solution was calculated and dissolved in the necessary volume of 1/1000 acetic or 0.01 M hydrochloric acid. The ACE inhibitor acid solution was then mixed with collagen and 2:1 DMEM: NaOH. Hydrogels were prepared in the same way as in section 2.3.2. The volume of 1 M NaOH used to raise the pH of the collagen solution was adjusted to account for the change in pH caused by the ACE inhibitors.

2.4 Mechanical testing of collagen hydrogels

2.4.1 Mechanical testing in compression

Gels for mechanical testing were prepared and tested in 24 well plates. Collagen solution was prepared as in section 2.3.2 with or without cells, and with or without ACE inhibitors. A 5 mm thick gel with a 16 mm diameter was produced by adding 1 ml of collagen solution to each well of a 24 well plate. After setting in an incubator (37 °C, 5 % CO_2) for 2 hours, 0.5 ml of complete DMEM was added to each well. The gels were maintained in the incubator until testing to maintain temperature and pH.

The method for mechanical compression testing developed by Busby and colleagues (2013) was adopted. Immediately prior to testing, the excess DMEM was removed from the well and the edge of the gel loosened from the sides of the well with a 2-

200 µl pipette tip. A porous platen, with a known permeability of 5.95 x 10^{-7} m^4/Ns and of the same diameter as the well, was attached to a 22 N load cell with a grip (Figure 2.2). The load cell was attached to either a Bose Electroforce 3100 or 3200 controlled by WinTest 7 software. The platen was lowered onto the gel surface until a preload of 0.01 N was achieved. The gel was allowed to equilibrate before being compressed by 0.25 mm (5% strain) at a rate of 0.025 mm s^{-1}. The displacement and load was recorded with Wintest 7 software. Stress was calculated and peak stress used to infer gel stiffness.

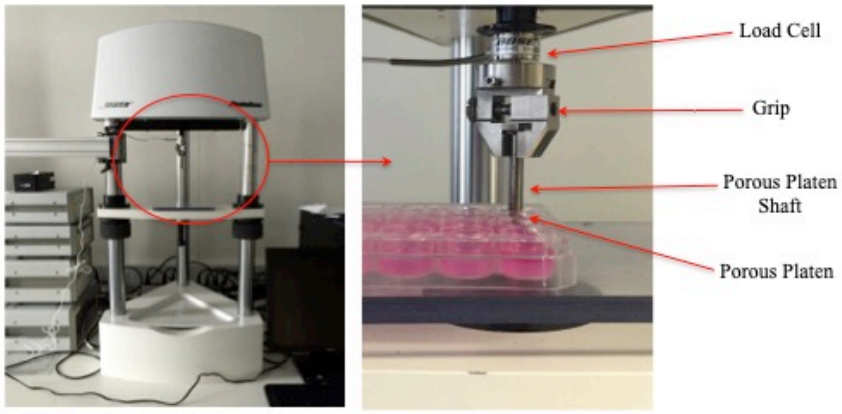


Figure 2.2: Collagen hydrogels in testing apparatus for confined compression mechanical testing. Sintered steel mesh porous platen attached with a grip to a 22N load cell and Bose 3200.

2.4.2 Mechanical testing in tension

Mechanical tension testing was adapted from a method developed by Saddiq and colleagues (2009). Collagen gels were prepared in specially designed casting plates. The casting plates were constructed from two Perspex sections fixed together with 6 nylon bolts: a solid plate and a plate with a rectangular hole, so the gel could be easily removed. A rubber gasket separated the two plates, which allowed the plates

to form a tight seal when fixed together (Figure 2.3). Before use, the plates and mesh (Figure 2.3 and Figure 2.4) were sterilised by UV light for 30 minutes, then soaked in 70% propanol and dried in a Class II laminar flow hood. The nylon diamond cut mesh was embedded in each end of the collagen gels to facilitate gripping by the test rig. Rectangles of 24 x 8 mm and 24 x 20 mm were cut from a diamond pattern nylon mesh (diamond apertures of 2.3 by 1.5 mm) and placed into the casting plate (Figure 2.4). Collagen solution was prepared as in section 2.3.2 and 8 ml added to the casting plate. The gel preparation method was later modified to improve the repeatability of the experiment as it was found that the mesh floated in the hydrogel while it was setting resulting in separation of the gel from the mesh. The new method involved creating a sandwich type gel by setting 8 ml of collagen solution in the casting plates then placing the mesh sections on the surface and covering them with a further 2 ml of collagen solution. The complete casting plate was placed in a 90 mm culture dish to maintain sterility and incubated (37 °C with 5% CO_2) for 2 hours. Complete DMEM was added to the gels once set and they were maintained in the incubator until required for testing.

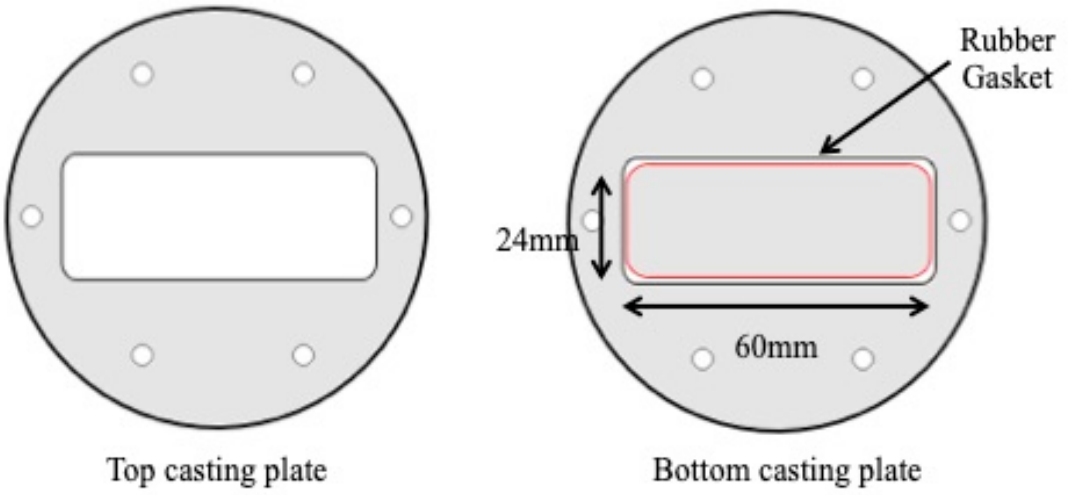


Figure 2.3: Diagram of casting plates for mechanically testing collagen gels in tension.

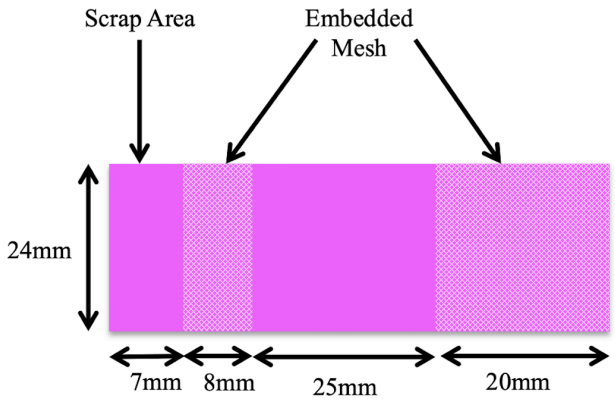


Figure 2.4: Gel preparation for mechanical testing in tension showing gel placement. Scrap area indicated as area used to manoeuvre specimen into testing rig.

For the mechanical testing, a specially designed and manufactured testing rig was employed (Figure 2.5). The fragile nature of the hydrogel meant that transfer of the gel from the casting plate to the testing rig proved difficult without damage. To avoid damage, the gel samples were floated into the testing apparatus. The upper casting plate was removed and the lower plate and gel submerged in a bath of sterile PBS

(phosphate buffered saline) (pH 7.4). The area of gel behind the mesh (scrap area labelled in Figure 2.4) was used to manoeuver the floating gel onto a specially designed spoon. The lower half of the testing rig (gel stage and lower grips) was then placed into the PBS bath, and the gel floated from the spoon into position. The upper grips were then fixed in place. Holders for the testing rig were designed (Figure 2.6) which prohibited lateral movement and attached the rig to the 22 N load cell and Bose Electroforce 3100 (TA Instruments, MN, USA) at each end. The gel stage was removed and the gel's thickness measured with a pair of digital Vernier callipers. The gel was subjected to a 4 mm extension at a rate of 1.66 mm/s, held for 20 seconds then returned to the starting position at a rate of 0.5 mm/s. A stress strain curve was plotted from the recorded figures, the initial plastic deformation of the gel was disregarded and the gradient of the linear section calculated to give a Young's modulus for the gel (Figure 5.3; 147).

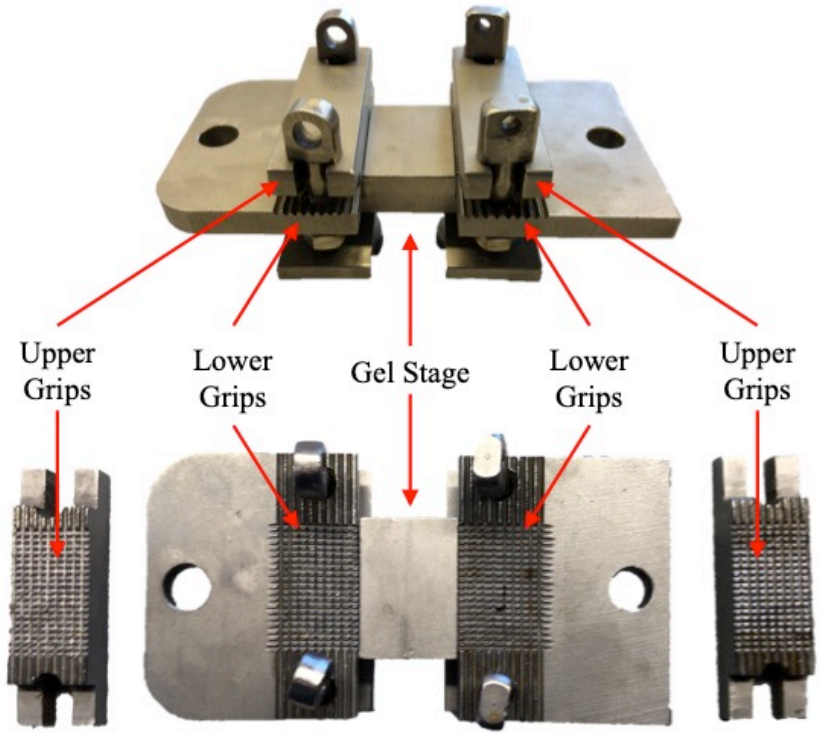


Figure 2.5: Mechanical testing rig to test in tension highlighting gel stage and upper and lower grips.

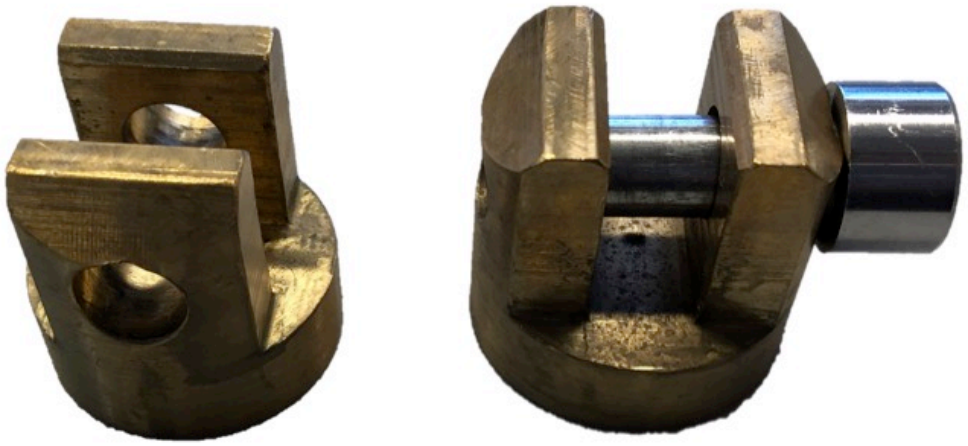


Figure 2.6: Holders designed to attach tension testing rig to Bose 3100 and 22 N load cell at each end. Holders were screwed into place and testing rig held in place with a pin.

2.5 Cell viability assays

2.5.1 MTT

An MTT assay was completed to measure cell viability on cells seeded either on tissue culture plastic, or within or on collagen gels. 10 mM MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) was prepared in PBS (pH 6.75), then sterile filtered. This could be stored at 4 °C for up to 2 weeks. The excess medium was removed and 0.5 ml MTT added to each well of the 24 well plate. The plate was incubated at 37 °C for 4 hours with the MTT solution. For assaying cells seeded in, or on, collagen gels, the MTT was removed and 0.5 ml of 0.5 % (w/v)

Type II collagenase (Gibco; catalogue number: 17101-015) in PBS (pH 7.4) added for 30 minutes after 3 hours 30 minutes of incubation with the MTT solution. The contents of each well were removed, added to separate eppendorf centrifuge tubes and centrifuged at 2000 rpm for 10 minutes, then the supernatant removed. For assaying cells seeded on tissue culture plastic, the MTT solution was removed thoroughly by pipette. To dissolve the MTT formazan, 0.5 ml of dimethyl sulfoxide was added and mixed thoroughly. The product was removed to a 96 well plate and the absorbance measured with a plate reader at 540 nm.

2.5.2 Staining Protocols

Two main staining protocols were employed to visualise cells: Live Dead staining and Actin staining.

For live dead staining, carboxyfluoro-diacetate (CFDA) and propidium iodide (PI) were used while phalloidin-flourescein isothiocyanate (phalloidin-FITC) was used to stain actin with a nuclear counterstain of 4,6-diamidino-2-phenylindole (DAPI).

For live dead staining with CFDA and PI, the medium was removed from cells or collagen hydrogels containing cells. Cells were rinsed twice with Dulbecco's PBS (DPBS; Catalogue Number BE17-513F, Lonza) at pH 7.4. One ml of PI (20 μg/ml PI in DPBS pH 7.4) was added to the cells and incubated in the dark for 1 minute. The PI was removed and the cells rinsed three times with DPBS pH 6.75. One ml of CFDA (25 μM in DPBS pH 6.75) was added and the cells incubated in the dark for 5 minutes, then the fluid was removed. Cells were then rinsed three times with DPBS

pH 6.75, and PBS added. Samples were examined with a Zeiss upright fluorescent microscope (Axioscope with ApoTome).

Actin was stained with Phalloidin-FITC and the nuclei counterstained with DAPI. Medium was removed, then cells were rinsed with warmed PBS. Formalin (4% in PBS) was used to fix the cells for staining at room temperature for 20 minutes. Cells on tissue culture plastic were rinsed three times with DPBS, or 5 times when in collagen gels. Phalloidin-FITC was added to cells at a dilution of 1/500 then incubated for 1 hour at 37°C in a dark, moist chamber. Cells were then washed 3 times with DPBS, for 5 minutes per wash. If cells were to be counterstained, a solution of 286 nM DAPI in PBS was added for 30 minutes. The stain was removed, cells rinsed and PBS added to examine with a Zeiss fluorescent microscope (Axioscope with ApoTome).

Chapter 3

Comparison of collagen from different sources

3 Comparison of collagen from different sources

3.1 Introduction

Collagen is the primary protein of the extracellular matrix in most species and the main structural protein in skin, bone and tendon (Kadler et al., 2007). It is a widely used biomaterial for both research purposes and medical applications. Grand View Research estimated the global market for collagen volumetrically was 920 tonnes in 2019, and is expected to grow with ever increasing applications. The healthcare industry was the biggest consumer of collagen by volume in 2019 and it is expected to account for 48% of the market volume in 2027 (Grand View Research, 2020).

The ready availability, high biocompatibility, biodegradability and low antigenicity of collagen make it a very attractive material for both *in vivo* and *in vitro* applications (Parenteau-Bareil et al., 2010). Studies using low concentration collagen hydrogels have been used widely both to increase understanding of cell behaviour and to increase cell viability in culture (Caliari and Burdick, 2016). The hydrogel creates an ideal 3-D environment which mimics the native ECM. Acid-solubilised rat-tail collagen (ASRT) is very popular in a laboratory setting as it is cheap and easy to produce in-house and sets consistently and reliably at a neutral pH of 7.4 at room temperature or above. Pepsin solubilised bovine collagen (PSB) is popular for medical research as it is suitable for use *in vivo.* Medically, collagen can be used in skin wound treatments, eye shields, bone substitutes, drug delivery systems and tissue constructs. Although both preparations have been extensively studied separately, this work sought to understand how the properties of ASRT collagen hydrogels used in research situations could be compared to those of PSB collagen

designed for medical purposes and to establish how results obtained from experiments using ASRT collagen could be translated from research to medical applications.

3.1.1 Sources of collagen

Type I collagen is a highly organised protein, present in a wide variety of species (Figure 1.1). It has a highly conserved structure as it appeared very early in terms of evolution (Lynn et al., 2004; Hudson et al., 2014). All tissues, regardless of species, containing type I collagen have the same primary amino acid sequence. However, crosslinking patterns vary between tissues within a single species and between species, giving rise to a wide variety of structures and functions (Hudson et al., 2014). Extraction of collagen for medical and research purposes is possible from a variety of sources: from simple marine life to complex mammals and from skin to tendon. Weak antigenicity and abundance make it highly attractive and highly available for extraction (C. H. Lee et al., 2001). Commercially, collagen is commonly extracted from bovine, murine, porcine, marine and avian sources (Bannister and Burns, 1972; Friess, 1998; Kittiphattanabawon et al., 2010; Zhang et al., 2011). In 2019, 35% (volume) of the global collagen market was sourced from bovine sources (Grand View Research, 2020). Bovine raw material resources are widely available, making it more cost effective than porcine or marine collagen. ASRT remains the most popular collagen for in vitro research, with approximately 17,000 papers published on it between 2015 and 2018. It is not, however, suitable for use in medical applications in humans (Section 3.1.2) and so many collagen preparations have been developed for such uses, including pepsin solubilised collagen sourced from bovine corium and jellyfish. There are advantages and

disadvantages to different sources of collagen depending on the end product; religious restrictions impinge on collagen sourced from porcine and bovine sources while disease transfer such as bovine spongiform encephalopathy and Creutzfeldt-Jakob disease is also a concern (Silva et al., 2014). Type I marine collagen sourced from fish by-products and jellyfish are relatively new, but chemically and functionally should be identical to collagen from mammalian sources (Addad et al., 2011). Compared to collagen from bovine and porcine sources, transfer of disease and religious restrictions associated with collagen from marine sources are of less concern, but they are relatively expensive. Marine sourced collagen is expected to be the fastest growing sector in the industry (Grand View Research, 2020).

3.1.2 Method of collagen extraction

Methods of extraction of collagen are wide and varied; including acid, enzymatic, alkali and salt extraction (Miller and Kent Rhodes, 1982; O'Sullivan et al., 2006; Zeugolis et al., 2008). Acid extraction is mainly employed in the production of collagen from weakly cross-linked collagen sources such as from tendons, whereas enzyme extraction is useful for obtaining monomeric collagen from heavily cross-linked collagen such as that in PSB (Antoine et al., 2014). ASRT collagen remains the most commonly used for research studies as it is inexpensive, production is repeatable and it is straightforward to produce 'in house'. Medically, collagen is used for a plethora of applications such as wound dressings, surgical reconstructive material, artificial skin and bone grafts and post-surgical eye shields (C. H. Lee et al., 2001). For these purposes, pepsin-extracted collagen from bovine sources (PSB) is the preferred preparation as it is less immunogenic and carries a lower risk of pathogen transmission than ASRT (Chattopadhyay and Raines, 2014).

The main source of immunogenicity in extracted collagen preparations comes from the telopeptide regions of the collagen molecule (Glowacki and Mizuno, 2008; Walton et al., 2010). Telopeptides are short unwound sections of between 15 and 25 amino acids (Ellingsworth et al., 1986), present at each end of the triple helix of collagen monomers (Figure 1.1; Yadavalli et al., 2010). Pepsin solubilisation cleaves these sections, reducing highly cross-linked type I collagen to collagen monomers (Antoine et al., 2014), thus reducing the immunogenicity of the collagen. These short, disorganised regions are however also important in collagen fibril formation as they play an essential role as sites for crosslinking monomeric collagen into fibrils (Woodley et al., 1991). The majority of the mechanical strength of collagen is derived from its crosslinking. Pepsin solubilised collagen (atelocollagen) therefore has both advantages and disadvantages for use in medical applications, the advantage being its lowered immunogenicity but the disadvantage for many applications being reduced mechanical integrity due to its more monomeric structure. There are several available techniques to overcome this by introducing crosslinks and increasing mechanical strength (Table 1.2) but these methods often result in increased toxicity and/or reduced biocompatibility (Section 1.2.2.1).

3.1.3 Mechanical testing

Mechanical properties have been well established as an important factor in biomaterial and 3D scaffold engineering (Lo et al., 2000; Caliari and Burdick, 2016). Cells respond very differently to a scaffold depending on how stiff or compliant it is; for this reason, it is important to tune the mechanical properties of the artificial scaffold to be similar to those of the tissue being replicated. It is very difficult to

fully evaluate the mechanical properties of soft collagen hydrogels; full mechanical characterisation would have to include all types of deformation: tension, compression and torsion/shear over a reasonable time component (Antoine et al., 2014). In addition, the viscoelastic nature of collagen (Wagenseil et al., 2003) and the requirement to immerse the samples in fluid whilst testing over a longer time period brings further difficulties. Because of this, there are few groups able to complete this kind of testing. This also means that there can be little comparison drawn between literature from different methods of testing (Table 3.1). A lack of standardisation of preparation method adds to the incomparability of data. In this work, a method developed by Busby and co-workers (2013), as described in section 2.4.1, was employed to test bulk stiffness in compression. This method was sensitive enough to compare collagen samples of different concentrations and drawn from different sources within the study. Drawing comparisons between this work and literature values, completed under different testing conditions and with different machinery, would be very difficult without further testing and post-testing analysis, not available at the time of this work.

Table 3.1: Review of mechanical testing results of collagen hydrogels tested by various methods.

Type of Hydrogel	Collagen concentration	Type of testing	Characterisation	Reference
Type I acid solubilised rat tail collagen gel embedded with mouse embryonic myofibroblasts	0.8 mg/ml	Tension	Ultimate strength: 4.7 + 1.2 kPa	(Gildner et al., 2004)
Type I bovine dermal collagen	2.0 mg/ml	Confined compression	**2 min creep** Aggregate modulus: 318.3 Pa **5 hr creep** Aggregate modulus: 6.32 Pa	(Knapp et al., 1997)
Type I bovine dermal collagen	2.0 mg/ml	Shear rheology	Zero-shear viscosity: 7.4x x10^5 Pa s Shear modulus: 15.5 Pa	(Barocas et al., 1995)
Type I acid solubilised bovine dermal collagen	Range from 0.3 mg/ml – 3.0 mg/ml	Tension	**0.3 mg/ml** Linear modulus: 1.54 ± 0.507 kPa Failure stress: 0.541 ± 0.138 kPa **3.0 mg/ml** Linear modulus: 24.3 ± 4.16 kPa Failure stress: 9.54 ± 1.35 kPa	(Roeder et al., 2002)
Type I acid solubilised rat-tail collagen	Range from 0.2% - 0.4%	Confined Compression	0.3% Peak stress: ~280 Pa	(Busby et al., 2013)

Freeze-dried Type I bovine tendon collagen crosslinked by dehydrothermal treatment		Unconfined compression	Compressive modulus: 145 ± 23 Pa	(C. R. Lee et al., 2001)

3.1.4 Contraction of collagen hydrogels with fibroblasts

Contraction of free-floating fibroblast populated collagen lattices (FPCL) is a time served wound healing model (Bell et al., 1979). The usefulness of this model for recapitulating wound healing has been called into question (Kuhn et al., 2000), with some likening the process to scar formation (Carlson and Longaker, 2004). When low concentration collagen hydrogels are embedded with fibroblasts and allowed to float freely, the fibroblasts begin to contract the collagen network; expelling water and thus increasing the concentration of collagen during the process. The technique is widely used in cellular biology to create densely packed collagen networks embedded with fibroblasts for a variety of research purposes. Cell to cell interaction, cell to collagen binding, cellular movement through the ECM and cellular remodelling of the collagen fibrils are all essential in the contraction of hydrogels by fibroblasts, thus contraction indicates cell viability and migration (Ehrlich and Rajaratnam, 1990; Ehrlich and Rittenberg, 2000; Daniels et al., 2003; Dallon and Ehrlich, 2010).

A question of interest in establishing the relevance of laboratory based studies to medical application was whether free-floating FPCLs produced from atelocollagen

would contract in the same way as those prepared from telocollagen. When consulting the literature, despite the apparent importance of this question only two papers explicitly addressing the issue were found and they reached different and conflicting conclusions. Woodley and colleagues (1991) found that atelocollagen would not contract when subjected to embedded fibroblasts and surmised that this was because the telopeptides had been cleaved. Frey and colleagues (1995) found that atelocollagen would contract in the same way as telocollagen. Because of the dubiety surrounding this, an experiment was devised to investigate whether atelocollagen and telocollagen from the same species (rat) would contract in the same way to attempt to better understand whether any observed differences arose from the method of preparation. The medical gold standard collagen: atelocollagen sourced from bovine corium, was also included in the experiment to investigate whether species had an effect on the contraction of FPCLs.

3.1.5 Atomic force microscopy

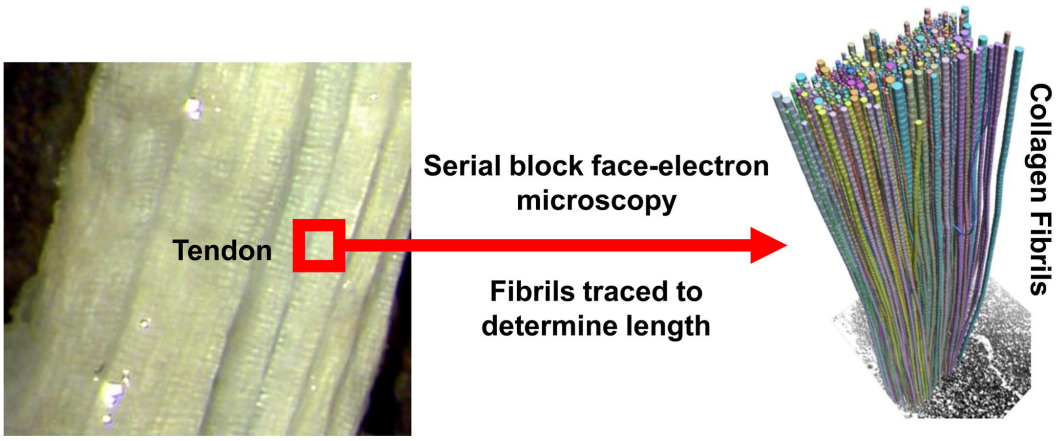
Atomic force microscopy is a useful technique which is able to provide 3D images of the surface of non-conductive samples, such as collagen. In this series of experiments it was used to visualise the surface of the collagen gel and to identify and measure banding patterns (Section 1.2.1; Figure 1.1), porosity and topography of the collagen gel surface. Collagen's distinctive 67 nm banding pattern is visible on the collagen fibril with AFM, a useful marker that collagen fibrillogenesis has taken place. Topography of the surface gave us an insight of collagen fibril diameter, which, when combined with the porosity gave an indication of the permeability of the gels. Permeability is an important factor in mechanical characterisation of the gels due to their biphasic nature. The initial resistance to deformation, when tested in confined

compression, is derived from fluid phase of the gel matrix structure, therefore increased permeability, allowing the fluid to move through the matrix more easily, is expected to reduce the relative stiffness of the gel.

3.1.6 Comparison of collagen from different species and different methods of extraction

Medical and laboratory preparations of collagen differ in several ways. For example, prior to extraction, collagen molecules in rat-tail tendons are weakly cross-linked and arranged in long parallel bundles, while in bovine corium, the collagen fibrils are arranged in a highly cross-linked basket weave (Berthod et al., 2001; Svensson et al., 2017). As a biomaterial, it is important that different collagen preparations are comparable in order that results can be translated from one preparation to another. Many laboratories use different preparations of collagen interchangeably. Understanding their properties facilitates reliable correlation of research results to the native collagen molecules.

(a)



(b)

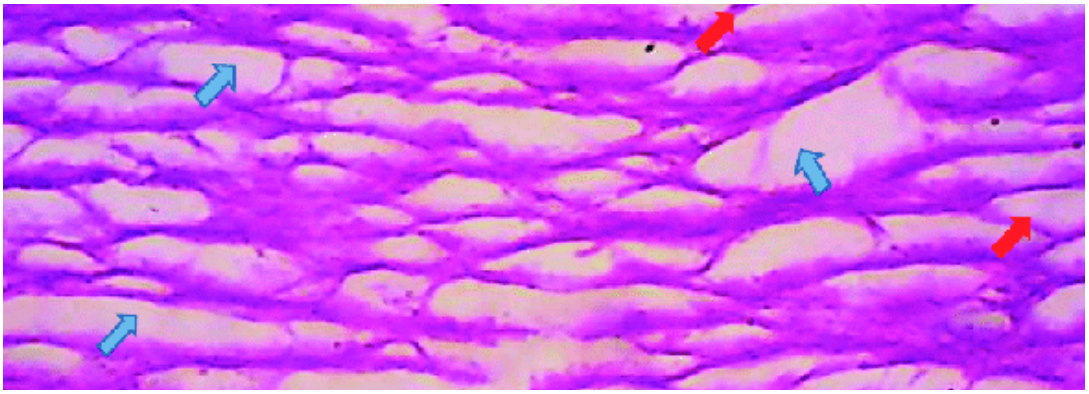


Figure 3.1: Collagen arranged in (a) parallel bundles in tendon (Svensson et al., 2017) and in (b) a basket weave structure in the dermis (Kp et al., 2018).

3.2 Methods

3.2.1 Materials

All chemicals, unless otherwise stated were purchased from Sigma Aldrich, UK. Rat-tails, for the preparation of acid and pepsin soluble rat-tail collagen, were kindly donated by Strathclyde University Biological Procedures Unit from control animals. Collagen Solutions Plc., Glasgow, kindly donated pepsin soluble bovine collagen.

3.2.2 Comparison of hydrogels of different collagen concentrations

The stiffness of collagen gels, prepared without cells, was measured to compare differences arising from the method of extraction and the species from which the collagen had been extracted. As described in section 2.3.1, rat-tail collagen was prepared by acid and pepsin solubilisation (Figure 2.1). Pepsin solubilised collagen from bovine dermis was obtained from Collagen Solutions Plc. For mechanical testing, collagen gels were prepared at concentrations of 0.2%, 0.3% and 0.4% from ASRT, PSRT and PSB collagen preparations. Gels were set for 2 hours as described in section 2.3.2 with a volume of 1 ml of collagen solution in each well of a 24 well plate. After setting, the gels were maintained in the incubator with 0.5 ml of DMEM for 24 hours. Mechanical testing in compression was then completed as described in section 2.4.1, based on the method described by Busby and colleagues (Busby et al., 2013). Briefly, gels were compressed by 5% strain at 0.5% s^{-1}. The resultant load on the gel was recorded and the stress calculated. Stress was plotted against time, peak stress was used to infer gel stiffness. Results were analysed by two-way ANOVA (Minitab Inc., State College, Pennsylvania, USA).

3.2.3 FPCL contraction

Gels of each collagen preparation (PSRT, ASRT and PSB) were prepared to test whether fibroblasts could contract each collagen type. Collagen gels were prepared as in section 2.3.3 with 5 ml of gel solution set in 60 mm petri dishes. Samples were prepared in four ways: with or without 3T3 fibroblasts (ATCC CRL-1658) and attached to the petri dish or free floating in medium.

The 3T3 cells were maintained in complete DMEM (as in section 2.2.2) in 75 cm^2 cell culture flasks. The gels were set for 1 hour 30 minutes at 37 ºC then polymerised by adding complete DMEM to the petri dish for a further 30 minutes. After polymerisation, the DMEM was removed and 3T3 cells were seeded on the surface of the gels at a seeding density of 3 x 10^4 cells/cm^2 (section 2.3.3).

The free-floating gels were detached from the petri dish with a sterile needle after polymerisation and transferred to a 90 mm petri dish to prevent reattachment. Enough medium was added to the plates to allow the gels to float freely. The DMEM was removed every 3 days from all gels and gels weighed and imaged from a fixed height. Fresh DMEM was added to the gels before returning to the incubator. The images were analysed to measure gel area using Image J software (U.S. National Institutes of Health, Bethesda, Maryland, USA).

3.2.4 Preparation, dehydration and AFM of collagen gels

The samples were prepared for microscopy (Figure 3.2) by a method adapted from Raub *et al*. (2007). Briefly, the gels were fixed with 4% formaldehyde then treated with 1% osmium tetroxide. Gels were serially dehydrated in graded ethanol and water (30%, 50%, 70%, 90% and 100% ethanol) before a serial dehydration of hexamethyldisilazane (HMDS) and ethanol (33%, 50%, 66% and 100% HMDS). The gels were dried on aluminium foil overnight. After drying, the gel was mounted on a glass slide and imaged with a stand-alone AFM (Oxford Instruments Asylum Research, Bio-MFP-3D, Santa Barbara, CA, USA) in contact and tapping mode to examine the collagen at a sub-micrometre scale. Resulting images were divided into a grid and fibril diameters from each square measured using Image J (U.S. National

Institutes of Health, Bethesda, Maryland, USA). The results were compared by one-way ANOVA followed by a Tukey honest significant difference (HSD) test using Minitab statistical analysis software (Minitab Inc., State College, Pennsylvania, USA).

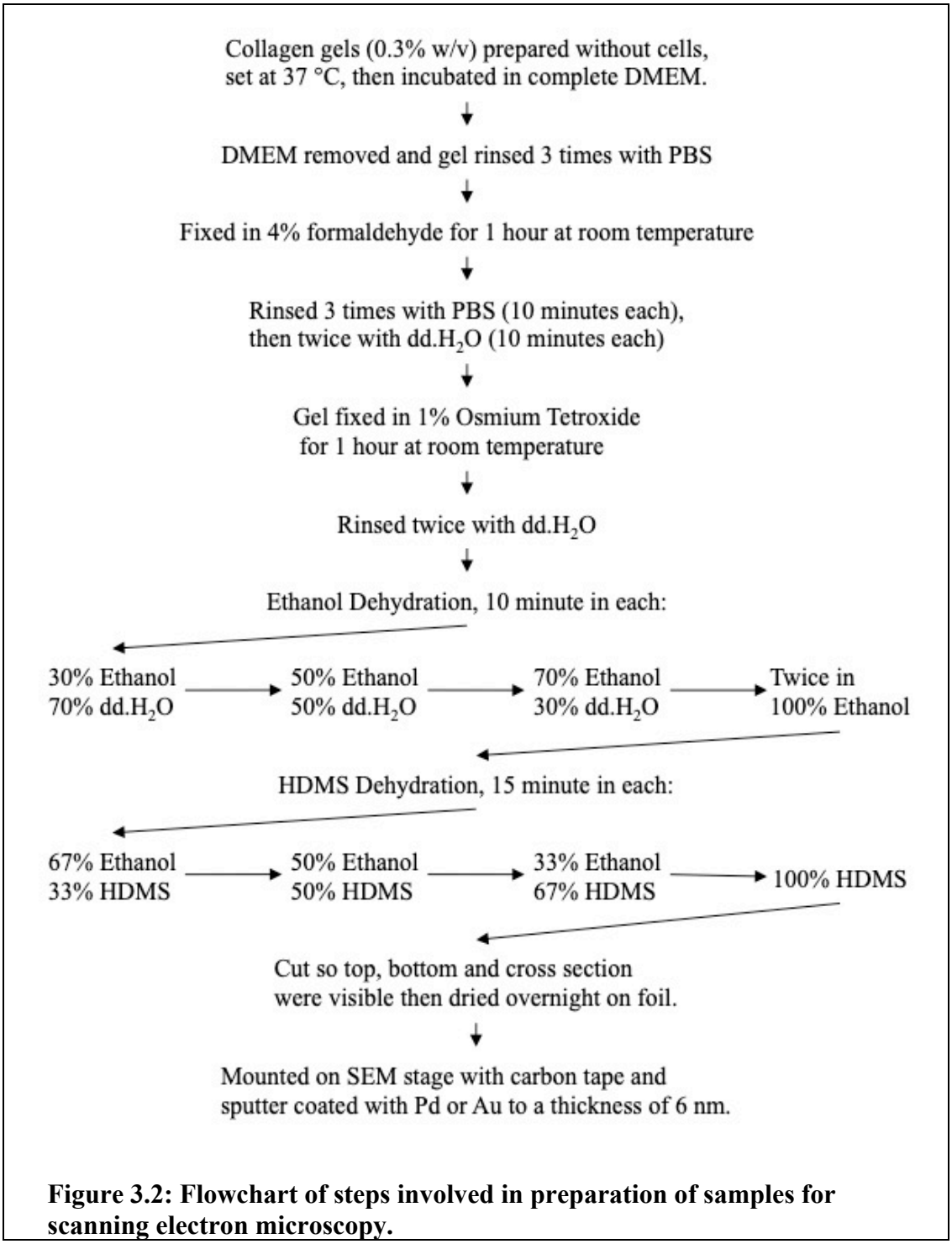


Figure 3.2: Flowchart of steps involved in preparation of samples for scanning electron microscopy.

3.3 Results

3.3.1 Mechanical testing: comparison of 0.2%, 0.3% and 0.4% ASRT, PSRT and PSB collagen.

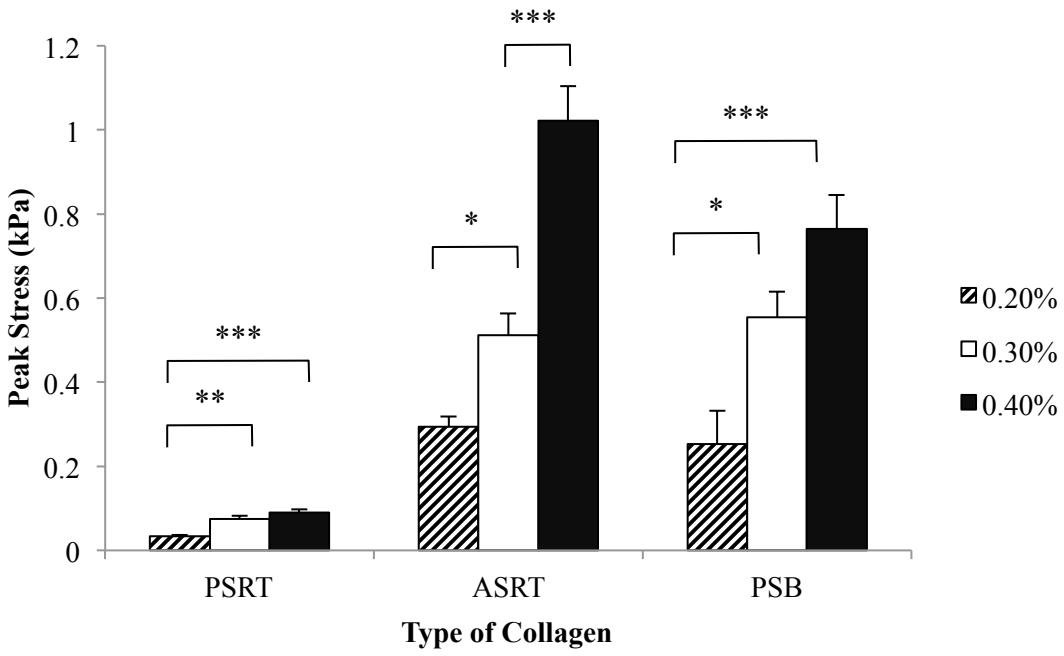


Figure 3.3: Comparison of mechanical stiffness of collagen gels produced from ASRT, PSRT and PSB collagen with collagen concentrations of 0.2, 0.3 and 0.4% (w/v). Results displayed as mean + S.E.M., n=6. Results compared by one way ANOVA followed by Tukey's test, * indicates p<0.05, ** p<0.01, *** p<0.001.

Mechanical testing showed that ASRT, PSRT and PSB all increased in stiffness with the increase from 0.2% to 0.4% collagen concentration in the gel (Figure 3.3). Within each collagen type, a one-way ANOVA was completed to test for any statistical difference between collagen concentrations. The results are shown on Figure 3.3. For PSRT, 0.2% gels were significantly more compliant than 0.3% gels ($p<0.01$) and 0.4% gels ($p<0.01$). There was a significant difference between each concentration of ASRT collagen, with 0.3% gels being significantly stiffer than 0.2%

gels ($p<0.05$) and 0.4% gels significantly stiffer than 0.3% gels ($p<0.001$). 0.2% PSB gels were significantly more compliant than 0.3% gels ($p<0.05$) and 0.4% gels ($p<0.001$).

Results showed that at 0.2% collagen concentration, ASRT collagen (0.29 kPa) was the stiffest, followed by PSB (0.25 kPa) then PSRT (0.034 kPa). At 0.3% collagen content, PSB was slightly stiffer (0.55 kPa) than ASRT (0.51 kPa) while PSRT (0.075 kPa) was much more compliant. At 0.4% collagen content, ASRT (1.02 kPa) was stiffest followed by PSB (0.77 kPa) then PSRT (0.09 kPa).

To compare the main effects of type of collagen and collagen concentration and the interaction of collagen type and concentration on the peak stress of the gels, a two-way ANOVA was completed. Type of collagen included ASRT, PSRT and PSB and concentration included 0.2%, 0.3% and 0.4% collagen. Both type and concentration had significant effects ($p<0.0001$) in addition to the interaction of the 2 factors ($p<0.0001$) on peak stress.

When a one-way ANOVA of all results for each collagen type were carried out, the results for PSB and ASRT collagen were not significantly different ($p=0.5756$) while PSRT was significantly different from both ASRT and PSB ($p<0.0001$). Overall, ASRT gels were found to be stiffest, although not significantly stiffer than PSB gels, while PSRT gels were most compliant.

3.3.2 FPCL contraction with different preparations of collagen

Attached and free-floating hydrogels were prepared from each collagen type, ASRT, PSRT and PSB, in each case both with or without 3T3 fibroblasts. Free floating gels

of all collagen types, when seeded with fibroblasts, decreased in area and weight over time (Figure 3.4). Cell-free detached gels increased in area and decreased in weight slightly over time. When compared by a two way ANOVA, both time and type of gel as well as the interaction of time and type were found to have significant effect on gel weight and area ($p<0.0001$). The area of the gels seeded with cells (Figure 3.4 (a), (c), (e)) initially increased then started to decrease as time progressed. The area of the gels that did not contain cells initially increased then plateaued. Attached gels did not change in area throughout the experiment, however they reduced in weight slightly over the nine days (Figure 3.4 (b), (d), (f)). From the measurements of the areas of the detached gels, a rate of contraction or expansion was calculated (Figure 3.5). For gels seeded with 3T3 fibroblasts, rate of contraction for ASRT, PSRT and PSB was 1.12 ± 0.08 (mean ± S.E.M.), 1.03 ± 0.16 and 1.46 ± 0.07 cm^2/day respectively. For gels prepared without fibroblasts, the rate of expansion for ASRT, PSRT and PSB was 0.17 ± 0.04, 0.56 ± 0.09 and 0.96 ± 0.19 cm^2/day respectively. When compared by a one-way ANOVA followed by a post-hoc Tukey's HSD test, there was no significant difference between the rate of expansion of ASRT, PSRT or PSB gels. PSB gels expanded significantly faster than ASRT ($p<0.05$) when prepared without cells.

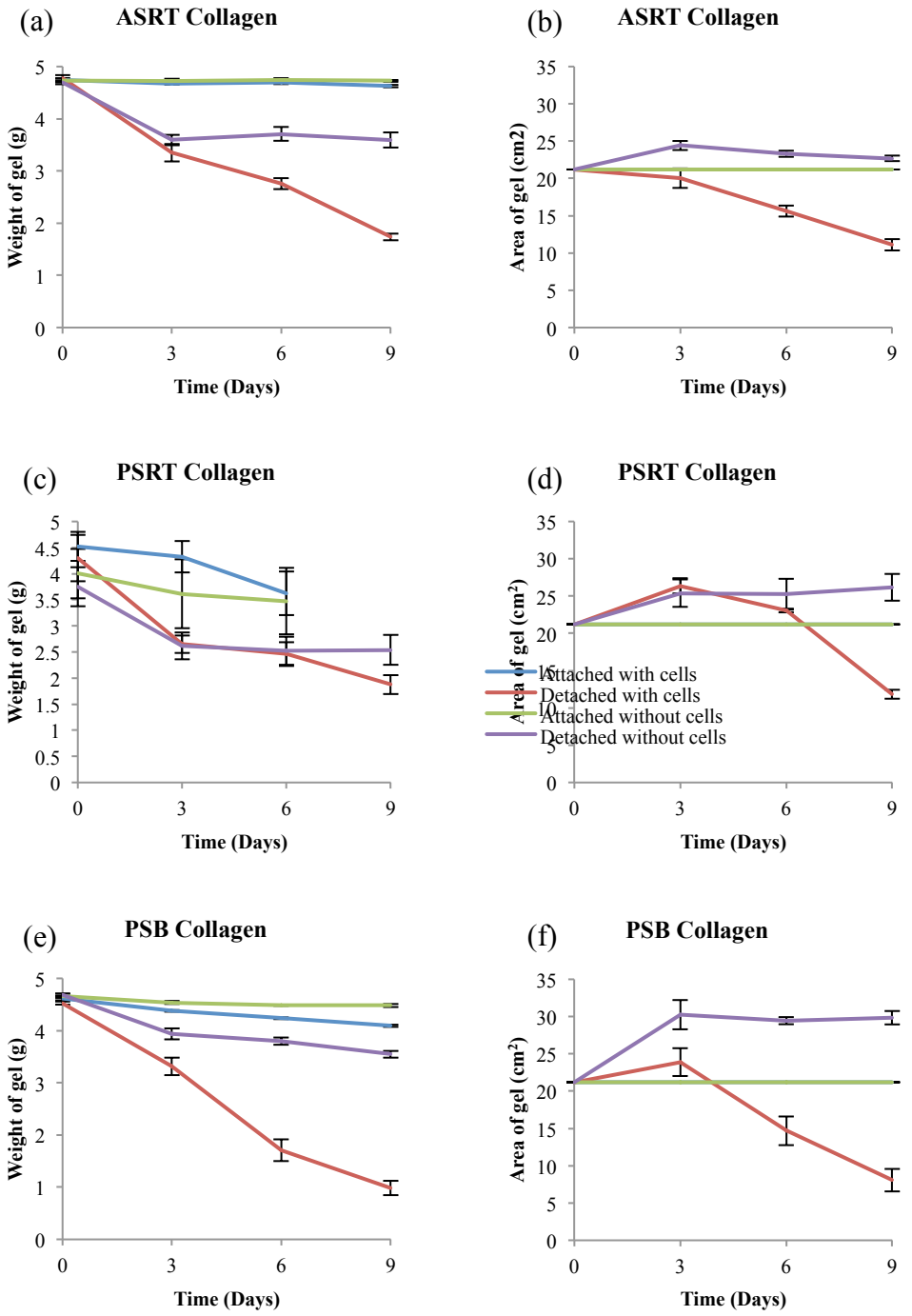


Figure 3.4: Comparison of fibroblast contracted collagen hydrogels prepared from ASRT (a,b), PSRT (c,d) and PSB collagen (e,f). (a, c, e) weight of gel against time as percentage of starting weight (b, d, f) area of gel against time as percentage of starting area of free floating hydrogels prepared with or without 3T3 mouse fibroblast cells. n=3, mean + S.E.M.

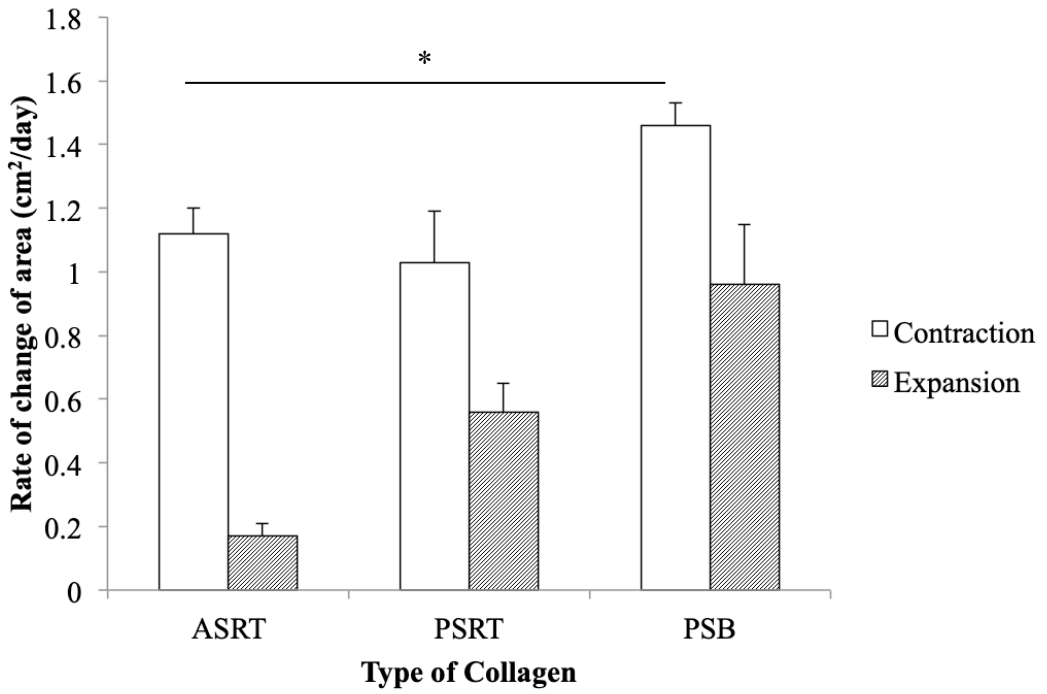


Figure 3.5: Rate of contraction or expansion of ASRT, PSRT and PSB collagen gels seeded with 3T3 mouse fibroblasts or prepared without cells respectively. n=3, mean + S.E.M. The rate of change was compared by a one-way ANOVA, followed by a post-hoc Tukey's HSD test, * indicates *p<0.05.*

3.3.3 AFM of dehydrated cell-free collagen gels

Collagen gels were prepared at a concentration of 0.3% (w/v) collagen without cells. Fibrils were examined by atomic force microscopy in contact mode to produce a 5 μm image; and in tapping mode to produce a 1 μm phase data image. The fibril diameters were compared by one-way ANOVA followed by Tukey's test which found significant differences ($p<0.001$) between each of the collagen types. The images are presented in Figure 3.7, with images (a,b,c) and (d,e,f) demonstrating height data and image (g,h,i) demonstrating phase data. The latter demonstrates the distinctive 67 nm banding of collagen, measured with ARgyle Light software

(Asylum Research, Oxford Instruments, California, USA). All types of collagen were found to have this distinctive banding in the phase data image (g,h,i), although the ASRT and PSRT collagen fibrils show greater clarity of banding in height data banding images (d,e). Figure 3.6 shows ASRT to have the thickest range of fibres (mean ± standard deviation, n=25) with a mean diameter of 0.159 ± 0.034 μm, followed by PSB with a mean of 0.138 ± 0.033 μm then PSRT with a mean of 0.114 ± 0.031 μm.

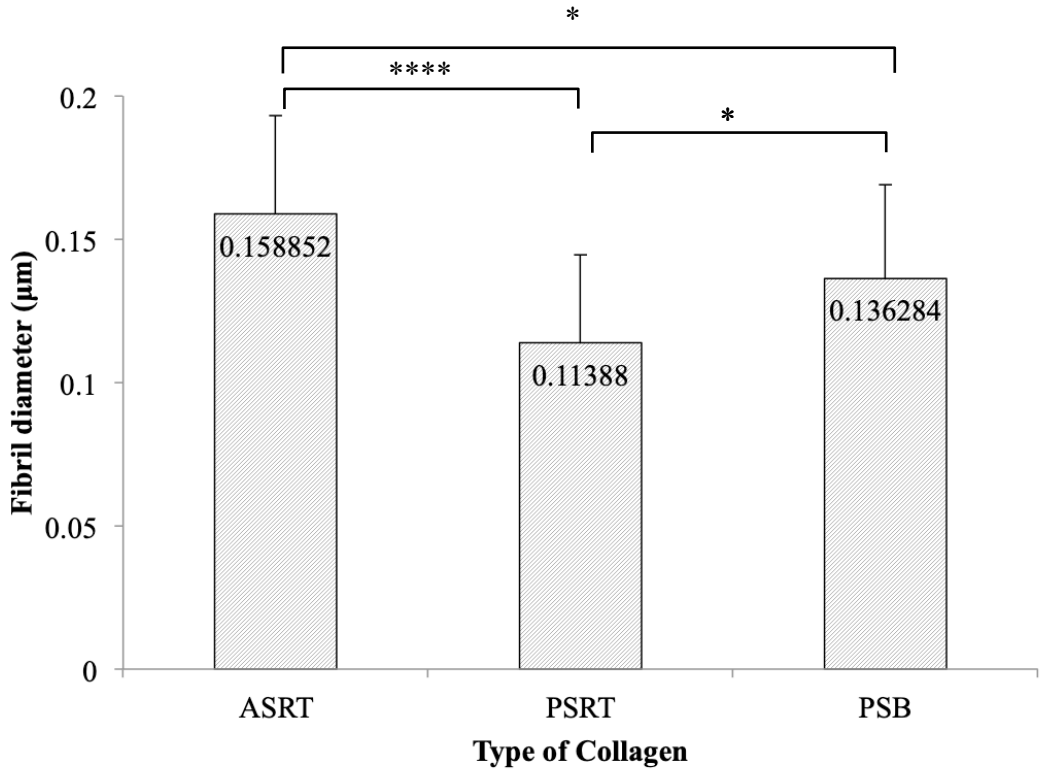


Figure 3.6: Fibril diameters of ASRT, PSRT and PSB measured by AFM; mean + standard deviation, n=25. Results compared by one-way ANOVA followed by a post-hoc Tukey's HSD test and all 3 found to be significantly different from each other (p<0.001). **p<0.05; **p<0.01; ***p<0.001; and ****p<0.0001.*

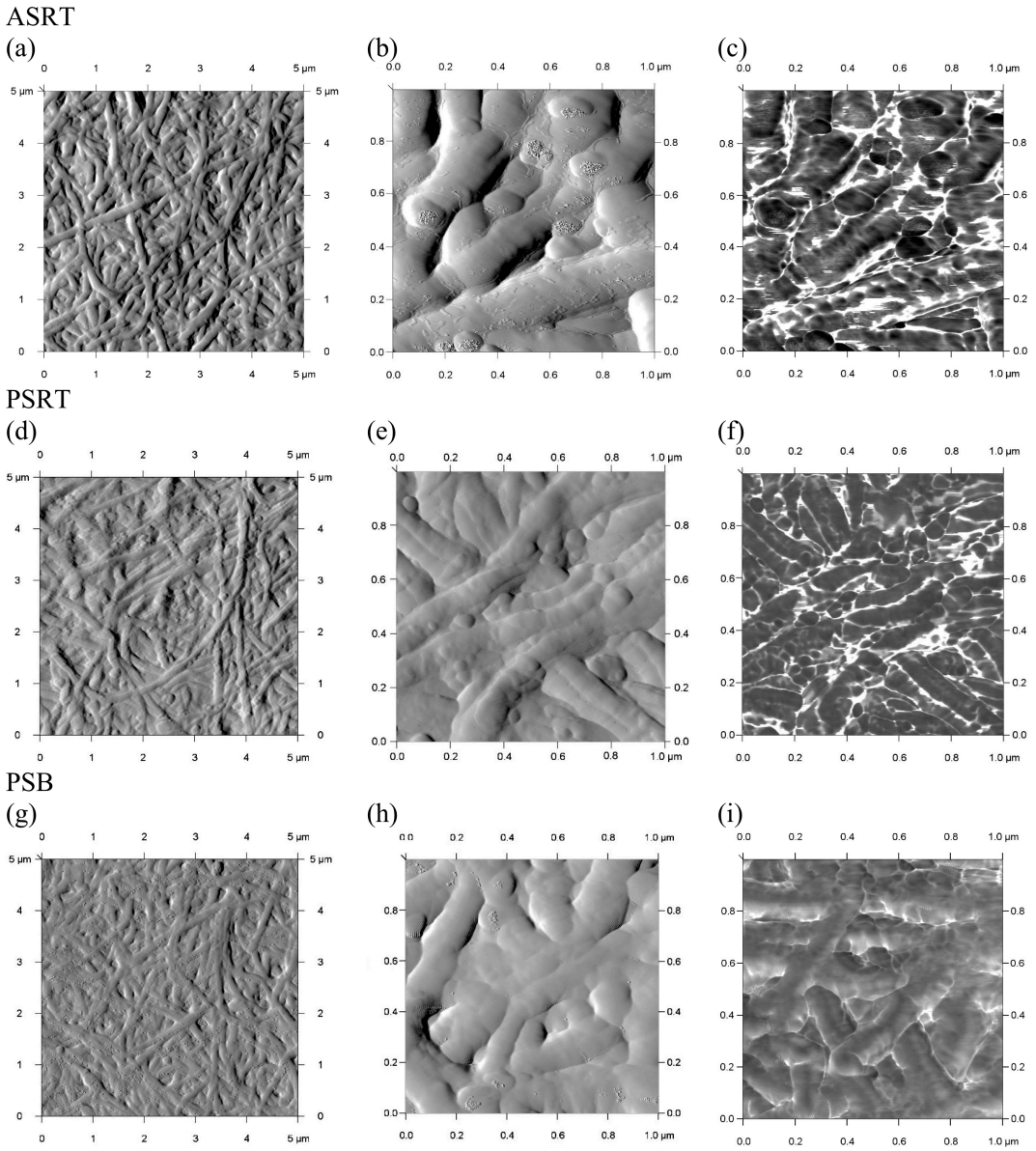


Figure 3.7: AFM images of dehydrated ASRT, PSRT, PSB collagen gels without cells. Range of images: (a), (d) and (g) 5 µm height data, z- range: 350 nm, (b), (e) and (h) 1 µm height data, z-range: 140 nm, (c), (f) and (i) 1 µm phase data, z-range: 90°.

3.4 Discussion

3.4.1 Confined compression of 0.2%, 0.3% and 0.4% collagen from acid and pepsin soluble rat-tail and pepsin soluble bovine dermis collagen

Mechanical testing compared three types of collagen: acid and pepsin solubilised rat-tail collagen and pepsin solubilised bovine collagen. ASRT is most commonly produced in-house for laboratory applications to improve cell viability by providing a tissue like substrate while pepsin soluble bovine collagen is a commercially available medical grade collagen suitable for use in humans. The cost of medical grade collagen is prohibitive in a laboratory setting so the mechanical properties of collagen prepared in-house were compared to those for commercially produced collagen to assess how they differed to inform how experimental results obtained using one collagen type from could be translated to the other in future applications. This is particularly important, as ASRT is not suitable for use in humans. There has been little work to compare how the properties of different collagen types differed directly. Neither has there been much work to compare how pepsin solubilisation affects the collagen's macroscopic structural and functional properties compared to acid solubilised collagen. As well as the normal lab standard ASRT collagen and medical grade PSB collagen, pepsin soluble RT collagen was prepared to compare how pepsin solubilisation affected the collagen prepared from the same tissue and species.

Collagen hydrogels are a biphasic material consisting of a solid phase (a network of collagen fibrils) and a fluid phase (interstitial fluid). Different methods of testing inherently test different aspects of the collagen gels' mechanical properties. Testing

the gel in tension mainly tests the properties of the solid element of the gel as all strain is taken up by the collagen fibril network; shear stress is borne by both the solid and liquid phases as both deform at same time with no interstitial fluid flow; compression primarily tests the fluid phase of the gel. Confined compression using a porous platen, as described in these experiments, measures the properties of both the fluid and solid phase, as the initial compression is taken up by the incompressible fluid which then begins to flow through the platen, thus creating an interstitial flow (Knapp et al., 1997).

Different measurements are also more or less useful depending on the properties being studied and the intended application of the gel. Rheometry is a method by which shear stress is applied to a sample to measure deformation. This can be considered less useful in delineating the responses of the fluid and solid phases, as the shear is applied to both phases concurrently. This may also be of less practical interest in this application, as scaffolds are unlikely to face much shear stress in normal usage. Tension testing of a hydrogel is extremely difficult due to the gel's inherent mechanical weakness, is expensive and only really tests the properties of the solid network, not the interaction with the fluid phase. Indentation or AFM tests properties on a micro scale, which is useful for cellular level mechanical testing, however can only test a small area of scaffold and is not conducive to high throughput.

Testing took place within a standard 24 well tissue culture plate. This limited disruption to the gel and maintained mechanical integrity before testing. Peak stress was used to infer stiffness or compliance of the gels with greater stress indicating

higher gel stiffness. The mechanical testing results show little difference in the stiffness of the ASRT and PSB gels ($p=0.576$), however, both are significantly stiffer than the PSRT gels ($p<0.0001$). Comparing the results between telocollagen (acid solubilised collagen with intact telopeptides) and atelocollagen (pepsin solubilised collagen with cleaved telopeptides) both sourced from rat tail tendons suggests telocollagen produces stiffer hydrogels in general. The gels from the acid preparation of rat tail tendons is significantly stiffer than the gel prepared from the pepsin preparation of the same source ($p<0.0001$).

As noted above collagen hydrogels can be described as a biphasic material consisting of a solid and a fluid phase. They are formed from a network of connected collagen fibrils and a large volume of interstitial fluid (Busby et al., 2013). Compressive load is initially supported by the fluid, then transferred to the collagen fibrils with deformation (Chandran and Barocas, 2004). Gel stiffness is therefore dependent on both the stiffness of the collagen network itself, and the flow of fluid through it, i.e. its permeability. A reduction in permeability increases the gel's apparent stiffness, as it is harder for the fluid to move out of the network so offers more resistance to deformation. Thicker collagen fibres would be expected to decrease permeability resulting in increased stiffness. These results are consistent with our AFM results (Figure 3.7), as the thickest collagen fibres (least permeable gel) are present in the ASRT preparation while PSRT gels have the thinnest fibres (Figure 3.6). These differences led to increased stiffness of ASRT gels while PSRT gels were most compliant (Figure 3.3).

Sato *et al.* (2000) measured the fibril thickness of acid and pepsin solubilised collagen from neonatal bovine skin by electron microscopy and the strength of the gels prepared from both materials by a modified viscosity measurement. A lead ball was placed on a collagen gel of each type, and the concentration of collagen required to support the ball was deduced. They found that pepsin-solubilisation produced weaker collagen fibrils than acid-solubilisation, with double the concentration of pepsin soluble collagen (0.25 mg/ml) required to support the ball compared to acid soluble collagen (0.125 mg/ml). They suggested this was due to the removal of telopeptides. Telopeptides are important sites of crosslinking and therefore important in collagen fibril assembly (Kadler et al., 1996). Removing telopeptides by solubilising collagen with pepsin consequently reduced available crosslinking sites, leading to thinner collagen fibrils. Our findings are consistent with this theory, as pepsin solubilised collagen produced thinner collagen fibrils.

Another factor believed to affect gel stiffness is the oligomeric content of acid-solubilised collagen. When collagen is solubilised by acid or pepsin, a mixture of collagen monomers (single collagen molecules) and cross-linked oligomers (2 or more cross-linked collagen molecules) is obtained. Kreger and colleagues determined that acid-soluble collagen preparations contains a higher proportion of oligomeric collagen compared to pepsin-solubilised preparations by SDS-PAGE (Kreger et al., 2010). Oligomeric collagens provide nucleation sites in fibril formation thus producing fibrils with increased stiffness (Bailey et al., 2011). Although this work did not characterise the monomer/oligomer content of the collagen molecules, we would assume they would correlate with Kreger and

colleagues' findings as we would expect a higher monomer content in the pepsin soluble collagen gels, leading to a more compliant gel, which is consistent with our experimental findings. It is well known that cross-links present in collagen fibrils are important factors in both mechanical strength and resistance to degradation by MMPs (van der Slot-Verhoeven et al., 2005).

The decreased permeability of the gel due to thicker collagen fibrils could be the cause of the increased stiffness of both the PSB and ASRT gels compared to the PSRT gels. Mechanical testing of individual collagen fibrils from each collagen type could be undertaken to confirm this (van der Rijt et al., 2006).

Based on the rat-tail collagen results, where acid solubilisation produced thicker fibrils and a stronger gel than pepsin solubilisation, we would expect that acid-solubilised collagen from bovine corium would produce a stiffer hydrogel than PSB, and thus ASRT. Collagen fibrils in bovine corium may be inherently stiffer than those present in rat-tails. Alternatively, the basket weave layout of the highly cross-linked collagen present in skin could itself be a significant factor in determining the stiffness of the gel as the basket weave may itself result in a stronger structure than that of the weakly cross-linked collagen bundles present in tendon. Further work to compare tissue source, i.e. rat-tail tendon versus rat-tail corium, as well as species, i.e. rat versus bovine corium, would be required to confirm whether tissue or species is responsible for the difference in stiffness. It is difficult to draw definitive conclusions from a single mechanical testing method as no method characterises all elements of the hydrogel. Figures reported in literature vary widely for the stiffness

of collagen hydrogels, depending on the method of testing employed. Differences in sources of collagen and preparation methods also have a significant effect.

An important result from these experiments for translating laboratory results to medical applications is that gels produced from ASRT collagen exhibited a similar stiffness to PSB collagen gels. Matrix stiffness is an important factor in the morphology and behaviour of cells (Park et al., 2011; Mason et al., 2013) embedded in or on a substrate. For practical applications, any difference in cell behaviour or morphology on the two substrates would not be expected to be a result of the hydrogels' stiffness, as the measured stiffness of ASRT and PSB hydrogels is extremely similar (Figure 3.3).

3.4.2 FPCL contraction with different preparations of collagen

Contraction of free-floating collagen hydrogels *in vitro* by embedded fibroblasts is a time served model of wound healing, initially described by Bell and colleagues over 40 years ago (Bell et al., 1979). Its use as a model of wound healing has been widely debated and the question is raised often as to whether it is a better model of wound healing or scar formation. Literature suggests that contraction of free-floating FPCLs is over-simplified in modelling granulation tissue (Kuhn et al., 2000; Carlson and Longaker, 2004). Wound healing is a complex process, comprising distinct phases of healing including angiogenesis, formation of new ECM and re-epithelisation completed by multiple cell types over time (Broughton et al., 2006; Velnar et al., 2009), rather than simply contraction of the wound as shown by this model. Many factors contribute to the progression and time of contracture of FPCLs including cell density, time point of release from tension, fibroblast or myofibroblast seeding and

concentration of collagen as these affect the mechanism of contraction i.e cell contraction, migration or elongation (Dallon and Ehrlich, 2008). There has been some suggestion that simply modelling contraction of collagen gels by fibroblasts better replicates fibrosis formation, with over contraction of the wound and excess collagen deposition displayed. Although reliance on contracted fibroblast populated collagen gels as wound-healing models has reduced, their use in laboratories worldwide has not diminished over time. They are still widely used in many areas of research, including cancer research (Cukierman et al., 2002; Abhilash et al., 2014) and in preparation of cell scaffolds for use *in vivo*. Additionally, cell contraction is a useful method of increasing collagen content in highly hydrated scaffolds. Frey and colleagues suggested fibroblast contraction was a useful measure of quality of collagen from different species, batches and preparation methods (Frey et al., 1995). As well as being a simple measure of cells ability to migrate, remodel and proliferate in FPCLs, the ability to contract collagen hydrogels is desirable for many research applications. In this work, the gels were seeded atop with 3T3 fibroblasts before being moved to larger dishes and floated in DMEM. Similar results were reflected in all preparations of collagen with the gels without cells increasing in area and decreasing slightly in weight. Low concentration collagen hydrogels are extremely compliant thus incapable of supporting their own weight whilst free floating. Self-compression leads to an increase in area, expunging water in the process shown as reduced gel weight (Figure 3.4). Gels seeded with cells all decreased in area and weight consistently as the fibroblasts contracted the gels (Figure 3.5). Questions raised during discussions at a meeting of Medical Research Scotland PhD researchers in 2016 led to investigating whether atelocollagen extracted with pepsin

would contract whilst free-floating and seeded with fibroblasts. Despite conflicting reports in the literature as to how they behaved (Woodley et al., 1991; Frey et al., 1995), our experiments found that atelocollagen gels did contract and found no difference in the contraction of the atelocollagen and telocollagen gels. Comparing atelocollagen (pepsin solubilised) and telocollagen (acid solubilised) from the same species and tissue, atelocollagen contracted more than collagen gels prepared with intact telopeptides however took longer to initiate contraction. This is an interesting result as it agrees with Frey and colleagues findings that atelocollagen would contract more than telopeptides however did not align with Tschumperlins suggestion that contraction of atelocollagen happened faster than telocollagen (Frey et al., 1995; Tschumperlin, 2013). Tschumperlin (2013) concluded that MMP-1 was required in migration of fibroblasts through acid solubilised collagen, where it was not required in pepsin solubilised collagen, thus initiation of contraction was expedited. Whilst Woodley (1991) and others suggested that contraction by the fibroblasts was reliant on telopeptides acting as anchor points for the fibroblasts, this work suggests either that pepsin solubilisation does not completely remove the telopeptides from the collagen or there are other sections of the collagen molecule involved in the contraction of the gels by fibroblasts.

3.4.3 Effect of collagen gel preparation on fibril formation

To directly compare the fibrils from each collagen preparation, AFM microscopy was used to compare fibril density and diameter and pore size of the collagen network. Alcohol dehydration was used to prepare the gels after fixation. Gels of around 5 mm thick dried into a thin layer less than 0.5 mm, and so only the surface layer of the gel could be examined although several layers of collagen fibrils were

present. No attempt to align the collagen had been made and so the fibres were randomly distributed. Large areas of the collagen were examined with the AFM in contact mode. To image single fibres, the area of interest became smaller, and the surface of the gel became sticky with repeated contact with the cantilever probe. Tapping mode was employed in imaging single collagen fibres as it was less damaging to the surface of the sample. Fibril diameter was compared by sectioning the image and analysing it with Image J image analysis software. The fibril diameters are presented in Figure 3.6. In all 3 types of collagen, the characteristic 67 nm banding of native Type I collagen (Chapman et al., 1990; Rainey et al., 2002) is clearly visible suggesting monomeric collagen has formed native fibrils. Their diameters ranged from 70 to 220 nm. This was consistent with fibril formation of 50 to 300 nm rather than fibre formation (Parenteau-Bareil et al., 2010). Both the pepsin preparations of collagen produce less well-defined and thinner collagen fibrils than the acid preparation. This result is consistent with the literature and could be caused by fewer telopeptides being present in the gels prepared using pepsin extraction. As mentioned in Section 3.4.2, telopeptides are thought to play an important role in fibril formation. Interestingly, the relative mechanical stiffness of the different gels, with ASRT being stiffest, PSB being close but slightly lower and PSRT being significantly more compliant was reflected in the fibril diameters illustrated in Figure 3.6 which shows the thickest fibrils in the ASRT gels, closely followed by PSB and significantly thinner fibrils in PSRT. This corroborates the theory that the stiffness of the gel in compression is reliant on collagen fibre thickness. Thicker collagen fibrils should slow fluid flow through the matrix and provide greater mechanical support to the solid phase resulting in gels with increased bulk stiffness. Raub and colleagues

suggested a similar theory in that smaller pore sizes, caused by gelation of collagen at different pH, increased hydrogel stiffness (Raub et al., 2007).

3.4.4 Conclusions of comparison of different collagen preparations

Collagen from two sources: rat-tail tendon and bovine corium was isolated and prepared by either acid or enzyme solubilisation. Hydrogels were prepared from the resultant collagen solutions (ASRT, PSRT and PSB) and compared by: mechanical testing, examination of fibril formation and contraction of free-floating hydrogels by embedded fibroblasts. Both ASRT collagen, which had been prepared 'in-house', and commercially available PSB collagen produced hydrogels with a similar stiffness, contraction profile and with fibrils of similar diameter and appearance. This suggested that data obtained from collagen gels prepared from the laboratory standard, ASRT, could be readily extrapolated to applications requiring medical grade collagen. This could be a useful finding for tissue engineering laboratories working on translational research.

The inherent weakness of these low concentration collagen hydrogels makes the application of these gels to wound dressing development and tissue engineering fraught with difficulty. Further work to improve mechanical strength and stability was undertaken in Chapter 4 by inclusion of the drug class Angiotensin converting enzyme inhibitors in the hydrogels. In Chapter 5, an alternative method of strengthening the hydrogels was explored: dehydrating the hydrogels in a controlled fashion using a super-absorber, sodium polyacrylate.

Chapter 4

Manipulating properties of collagen hydrogels using ACE inhibitors

4 Manipulating properties of collagen hydrogels using ACE inhibitors

4.1 Introduction

4.1.1 ACE inhibitors in treatment of hypertension

ACE inhibitors are a commonly prescribed group of pharmaceuticals used to treat hypertension and congestive heart failure. They block angiotensin converting enzymes to inhibit the conversion of angiotensin I to angiotensin II (Figure 4.1). Angiotensin II, is the primary stimulus of the Renin-Angiotensin system, acting on Angiotensin receptor I and II to generate several biological effects including vasoconstriction and release of aldosterone (Fyhrquist and Saijonmaa, 2008).

Three different ACE inhibitors were used in these experiments: Captopril, Enalapril maleate and Lisinopril (Figure 1.3). These were chosen as they are readily available and extremely common in the treatment of high blood pressure with over 44 million community prescriptions issued for ACE inhibitors in England in 2018 (NHS Digital, 2019). They also differ in their chemical structures, giving an array of structural difference for investigation. Captopril was chosen as it was the first orally available ACE inhibitor (Robertson, 1994). It has been largely replaced in its use in patients because of various side effects, such as a metallic taste and a skin rash, which were attributed to its sulfhydryl group. The second ACE inhibitor developed was Enalapril maleate, a pro-drug developed to overcome the side effects of captopril caused by the sulfhydryl group. This made enalapril the first non-sulfhydryl ACE inhibitor and also the first pro-drug ACE inhibitor. Enalapril is on the World Health Organisations list of essential medicine as both an anti-hypertensive and a medicine used in heart

failure (World Health Organization, 2019). It is an ester of the active metabolite enalaprillat and ethanol, which makes it orally available with fewer side effects than captopril (Packer et al., 1986). Lisinopril is a lysine analogue of enalapril, which makes it an orally available pharmacologically active drug.

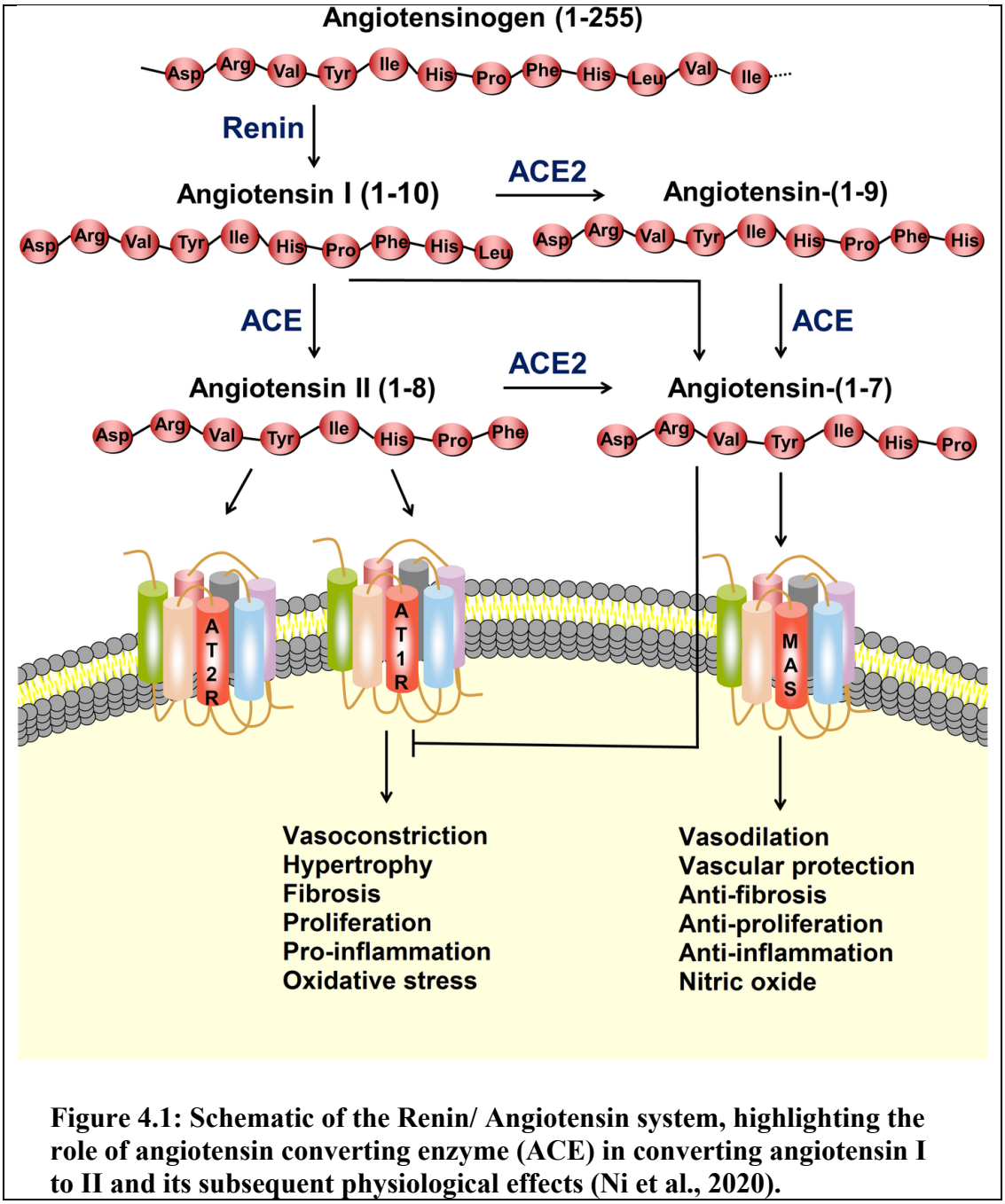


Figure 4.1: Schematic of the Renin/ Angiotensin system, highlighting the role of angiotensin converting enzyme (ACE) in converting angiotensin I to II and its subsequent physiological effects (Ni et al., 2020).

4.1.2 MMP inhibition by ACE inhibitors

Degradation and remodelling of the ECM are essential for organogenesis, tissue generation, wound healing and growth. The matrix metalloproteinases (MMPs) are a group of zinc-dependent endopeptidases responsible for degradation and regulation of the ECM as well as for releasing biologically active, membrane-bound proteins such as growth factors, chemokines, and cytokines (Klein and Bischoff, 2011). MMPs are regulated by Tissue inhibitors of matrix metalloproteinases (TIMPs) which act on MMPs by chelating catalytic zinc atoms (Jacobsen et al., 2010). Controlled MMP activity plays a critical role in organogenesis, wound healing and tissue remodelling in healthy tissues; however it can be the cause of many diseases if regulation fails, including, but not limited to, rheumatoid arthritis, congestive heart failure, myocardial infarction and cancer (Nagase et al., 2006). Butler and Overall (2009) suggest that MMPs have a much broader range of functions than previously thought. However, a detailed report on this is beyond the scope of this study. Humans have 23 MMPs, each of which acts on a specific target material, such as collagen, gelatin or elastin (Table 4.1). Most MMPs are released from cells to the extracellular matrix to degrade ECM and non-ECM proteins alike, although some are membrane-bound (Klein and Bischoff, 2011). A link between ACE inhibitor treatment and increased collagen synthesis was noted by Claridge et al (2004) and the treatment was thought to decrease incidence of myocardial infarction. As collagen production is a delicate balance of synthesis and degradation, the net increase in collagen could have been caused by either increased collagen synthesis or inhibited degradation. In 2007, Brower and colleagues found that treating CHF with ACE inhibitors limited left ventricle dilation, the primary cause of CHF. Dilation of

the left ventricle is primarily due to a breakdown of the collagen matrix by MMPs. McElmurray (1999) found that patients with congestive heart failure had increased expression of MMPs and Brower *et al*. hypothesised that ACE inhibitors reduced MMP activity resulting in reduced breakdown of the myocardial collagen matrix thereby limiting left ventricle dilation. Several other groups have since shown that ACE inhibitors could have inhibitory effects on MMP activity both *in vivo* and *in vitro* (Sorbi et al., 1993; Nguyen et al., 1994; Brower et al., 2007).

Table 4.1: List of MMPs, alternative names, sub-family and location. Table adapted from (Klein and Bischoff, 2011; Iyer et al., 2012; Karthikeyan et al., 2013; Djuric and Zivkovic, 2017).

MMP	Alternative name	Sub-family	Location
1	Collagenase-1; Interstitial collagenase	Collagenase	Soluble
2	Gelatinase A; type IV collagenase	Gelatinase	Soluble
3	Stromelysin-1; Transin-1	Stromelysin	Soluble
7	Matrilysin; Pump-1	Matrilysin	Soluble
8	Collagenase-2; Neutrophil collagenase	Collagenase	Soluble
9	Gelatinase B; 92 kDa type IV collagenase	Gelatinase	Soluble
10	Stromelysin-2	Stromelysin	Soluble
11	Stromelysin-3	Stromelysin	Soluble
12	Macrophage metalloelastase	Other	Soluble
13	Collagenase-3	Collagenase	Soluble
14	Membrane type-1 MMP	Membrane type MMP	Membrane-anchored
15	Membrane type-2 MMP	Membrane type MMP	Membrane-anchored
16	Membrane type-3 MMP	Membrane type MMP	Membrane-anchored
17	Membrane type-4 MMP	Membrane type MMP	Membrane-anchored
19	Human ortholog of *xenopus* MMP-18	Stromelysin	
20	Enamelysin	Other	
21	Human ortholog of *xenopus* xMMP	Unknown	
23	Cysteine array MMP; Femalysin; MMP-22	Other	
24	Membrane type-5 MMP	Membrane type MMP	Membrane-anchored
25	Membrane type-6 MMP; Leukolysin	Membrane type MMP	Membrane-anchored
26	Matrilysin-2/endometase	Matrilysin	Soluble
27		Unknown	
28	Epilysin	Other	

4.1.3 Role of ACE inhibitors in treatment of other diseases

ACE inhibitors have been used to target many diseases in addition to hypertension. Most commonly, ACE inhibitors are used in the treatment of CHF (McElmurray et al., 1999; Sakata et al., 2004) and in recovery from myocardial infarction (Pfeffer et

al., 1985; Pfeffer, 1998; Jin et al., 2007), however they can also be used in treatment of fibrosis and inflammation (Passos-Silva et al., 2015) and have been suggested as a treatment for some types of cancer and retardation of tumour growth (Egami et al., 2003; Lindberg et al., 2004; Passos-Silva et al., 2015; Srivastava et al., 2020)

4.1.4 Manipulation of collagen gel properties with ACE inhibitors

From the literature, ACE inhibitors seemed to have an inhibitory effect on MMPs. Being able to control the degradation rate of collagen is a desirable attribute in scaffold design. Degradation rate can be modulated to allow biomaterials to have a controlled drug release as the scaffold degrades, or to coincide with the synthesis of new ECM. Therefore, as the MMPs are understood to be the main agent of degradation in collagen matrices, inhibiting their activity and hence their effect could be a viable method of slowing degradation of the scaffold. An experiment was devised to test whether incorporating ACE inhibitors into collagen cell scaffolds could improve mechanical properties and/or decrease degradation. The three ACE inhibitors chosen for the study were captopril, enalapril and lisinopril. Each of the chosen ACE inhibitors were dissolved within collagen gels, prepared with and without cells. To establish if the ACE inhibitors had any effect on the scaffolds, or resident cell population several experiments were designed. The mechanical stiffness of the hydrogels was measured by compression, cell viability by MTT assay, elution of the drugs by HPLC-MS and finally cell behaviour by the wound healing model developed by Bell and colleagues (Bell et al., 1979). Slowing collagen degradation could, in turn, have a positive effect on mechanical properties of the collagen gels, as the integrity of the structure is retained for longer. Measuring contraction of collagen

gels populated with fibroblasts showed whether the ACE inhibitors affected the behaviour and viability of the fibroblasts. Also, inhibition of MMPs has previously been shown to reduce contraction of free-floating collagen lattices by embedded fibroblasts (Daniels et al., 2003).

4.1.5 Mechanical testing

The mechanical characteristics of cellular scaffolds provide strong mechanical cues in guiding cell behaviour and morphology (Discher, 2005). With this in mind, controlling the mechanical properties of cell scaffolds so as to mimic the native ECM is an important step cell scaffold design. Thus, developing accurate methods of measuring their mechanical properties is essential for good scaffold design.

4.1.5.1 Confined compression

The confined compression method developed by Busby and colleagues (2013) was chosen to measure bulk stiffness of collagen hydrogels. The method allowed gels to be tested within a standard tissue culture plate by a porous platen attached to a Bose Electroforce testing instrument. The gels could be prepared by a standardised method and drugs, cells or both could be included easily. Calculating peak stress from the load response and a prescribed displacement of the porous platen proved sensitive enough to show significant difference between the stiffness of collagen gels containing 0.1% differing collagen content (Busby et al., 2013).

4.1.5.2 Variability of compression results between machines

Mechanical testing was initially carried out using a Bose Electroforce 3200 testing instrument controlled by WinTest 7 software. A porous platen was attached to a 22 N load cell with a stainless steel grip, which was then attached to the testing instrument. During the course of the study, a fault occurred in the power supply of the Bose 3200. Although every effort was made to repair this, the fault proved irreparable and rendered the equipment unusable. The remainder of the mechanical testing had to be completed with a Bose Electroforce 3100 instrument. Ideally, these machines should have been calibrated to the same standard, however it soon became clear that this had not been completed, as, although the magnitude similar, control gels had very different values when subjected to the same tests on the Bose 3100 and 3200. This problem did not become apparent until the Bose 3200 had become unusable and as the mechanical testing was destructive in nature, the samples could not be retested. Ideally a non-biological sample could have been used to normalise the readings of each machine, however this was impossible. Therefore, the results obtained from control samples tested on each machine were used to normalise the results. These absolute results therefore cannot be translated from this study; however, we can confidently compare results within the study to measure differences in the properties of the gels to which cells and/or the ACE inhibitors had been added with the results from the control gels prepared without modulators.

4.1.6 Collagenase inhibitor and acetyl cysteine

To attempt to ascertain a mechanism of action of the ACE inhibitors on the collagen gels, acetylcysteine and collagenase inhibitor I produced by Calbiochem were

chosen. Acetylcysteine was chosen for two reasons: to mimic the effect of the sulfhydryl group in captopril to test its role in limiting cell growth, and as a similarly sized molecule to captopril to test the theory that the ECM was stiffened by the captopril molecules becoming physically lodged within the super helix structure of the collagen molecule. The collagenase inhibitor was chosen to compare with the properties of the samples to which the ACE inhibitors had been added to test the hypothesis that the ACE inhibitors reduced ECM degradation through inhibition of collagenase activity. The gels with acetylcysteine or collagenase inhibitor were prepared in the same way as the ACE inhibitor loaded gels. The same experiments were carried out as with the ACE inhibitor loaded gels: mechanical testing; FPCL contraction; and cell number over time; were carried out on test samples to which acetylcysteine or collagenase inhibitor had been added.

4.2 Methods

4.2.1 Materials

All materials, unless otherwise stated, were purchased from Sigma Aldrich, UK.

4.2.2 Preparation of collagen gels containing ACE inhibitors for MTT and mechanical testing

Two sets of identical collagen gels were prepared for mechanical testing and measuring cell viability. Both mechanical testing and MTT are destructive end point tests, so a complete set of gels were required for testing at each time point. For mechanical testing (as in section 2.3.2), briefly, 1 ml gels with a concentration of 0.3% collagen were prepared with or without ACE inhibitors or cells. The gels were

set for 2 hours in a 37 °C incubator, polymerised with complete DMEM and returned to the incubator until testing. The testing conditions are presented in Table 4.2. Fibroblasts from the 3T3 cell line were either mixed into the gel solution prior to setting (suspended cells) or seeded on the surface of the set gel (top seeded cells) after polymerisation with DMEM. The gels were maintained in complete DMEM until undergoing testing. Medium was changed on the gels every third day, testing was undertaken on day 0, 3, 6 and 9.

Table 4.2: Preparation conditions of collagen gels prepared with ACE inhibitors and cells, for mechanical testing and cell viability for each time point. Mechanical testing equipment used to test stiffness in compression included.

Experiment	Cells	Additive	Mechanical Testing
1	None	Control	Bose 3200
1	Top-seeded	Control	Bose 3200
1	Suspended	Control	Bose 3200
1	None	1mM Captopril	Bose 3200
1	Top-seeded	1mM Captopril	Bose 3200
1	Suspended	1mM Captopril	Bose 3200
1	None	1mM Enalapril maleate	Bose 3200
1	Top-seeded	1mM Enalapril maleate	Bose 3200
1	Suspended	1mM Enalapril maleate	Bose 3200
1	None	1mM Lisinopril	Bose 3200
1	Top-seeded	1mM Lisinopril	Bose 3200
1	Suspended	1mM Lisinopril	Bose 3200
2	None	Control	Bose 3100
2	Top-seeded	Control	Bose 3100
2	Suspended	Control	Bose 3100
2	None	Acetylcysteine	Bose 3100
2	Top-seeded	Acetylcysteine	Bose 3100
2	Suspended	Acetylcysteine	Bose 3100
2	None	Collagenase Inhibitor	Bose 3100
2	Top-seeded	Collagenase Inhibitor	Bose 3100
2	Suspended	Collagenase Inhibitor	Bose 3100

4.2.3 MTT activity of fibroblasts contained in collagen gels

An MTT assay was undertaken to measure cell viability in or on collagen hydrogels. The DMEM was removed from each gel and 0.5 ml sterile-filtered 10 mM MTT in PBS (pH 6.75) added before returning to a 37 °C incubator for 4 hours. After the incubation, all excess MTT was removed and 0.5 ml 0.5% (w/v) Type II collagenase

in PBS (pH 7.4) added to each gel. The gel was manually disrupted, by pipetting up and down, and incubated in a shaking incubator for 30 minutes. The contents of each well were added to a centrifuge tube and centrifuged at 2000 RPM for 10 minutes to pellet the cells. The supernatant was then removed, and the formazan re-suspended in 0.5 ml dimethyl sulfoxide. The coloured solution was then removed to a 96 well plate and absorbance measured at 540 nm with a plate reader.

4.2.4 Confined compression of collagen gels containing ACE inhibitors

Mechanical testing of collagen gels containing ACE inhibitors was completed in the same way as in section 2.4.1. Briefly, the medium was removed from the 1 ml hydrogels and the gel detached from the sides of the well with a pipette tip. The first experiment, measuring mechanical properties of control gels supplemented with captopril, enalapril maleate or lisinopril, used a Bose 3200; in the second experiment, control gels supplemented with acetylcysteine or collagenase inhibitor, were tested with a Bose 3100. The gels were tested within a 24 well plate to enable confined compression. A porous platen was used to compress the gels by 5% strain at a rate of 0.5 % s^{-1}. The Bose 3100 and 3200 were not calibrated to one another, so the data were normalised to the day 0 control figures for each experiment.

4.2.5 Preparation of collagen gels containing ACE inhibitors to measure fibroblast contraction

To measure fibroblast contraction of ACE inhibitor supplemented hydrogels, 4 groups of gels were organised in 6 ways (Table 4.3). 0.3% collagen hydrogels were prepared as in section 2.3.4. Five ml gels were prepared in 60 mm diameter petri dishes, and supplemented with ACE inhibitors as required. Suspended cell matrices

were prepared by adding 3T3 fibroblasts to the gel solution prior to setting to give a final cell density of $3x10^4$ cells/cm^2. Top seeded cell matrices were set, polymerised for 30 minutes with complete DMEM and seeded with $3x10^4$ cells/cm^2 (3T3 Fibroblasts; prepared as in section 2.2.3). Control gels were set for 2 hours then polymerised for 1 hour with complete DMEM. The free-floating gels were loosened from the sides of the 60 mm petri dish with a sterile needle and transferred to a 90 mm petri dish. All gels were weighed then imaged from a fixed height on graph paper. DMEM was added to all the gels and the free-floating gels gently agitated until they began to float. The gels were weighed, imaged and DMEM changed every 3 days. Image J software was used to measure area of gels from the images (Schindelin et al., 2012).

Table 4.3: Conditions of cell seeding and attachment of collagen gels containing ACE inhibitors investigating contraction of free floating collagen hydrogels.

		Cell Seeding		
		Control	**Top surface**	**Suspended**
Gel Supplement	**Control**	Attached	Attached	Attached
		Free-floating	Free-floating	Free-floating
	1mM Captopril	Attached	Attached	Attached
		Free-floating	Free-floating	Free-floating
	1mM Enalapril maleate	Attached	Attached	Attached
		Free-floating	Free-floating	Free-floating
	1mM Lisinopril	Attached	Attached	Attached
		Free-floating	Free-floating	Free-floating

4.2.6 High performance liquid chromatography-Mass spectrometry determination of ACE inhibitor concentration

Concentrations of ACE inhibitors in media were quantified by liquid chromatography-mass spectroscopy (LC-MS). A Kromasil 60-5 SIL 150x3.0 μm column was used with a combined Exactive Orbitrap LC-MS system (Thermo Fisher Scientific, Bremen, Germany). Aliquots (10 μl) of sample solution were injected into the column. Samples were eluted from the column at a flow rate of 0.3 ml/min. Two solvents of 90 % 20 mM Ammonium Acetate and 10 % methanol (A) and 90 % Acetonitrile and 10 % 20 mM Ammonium Acetate with an automatic gradient of 20:80 A:B (v/v) to 70:30 A:B (v/v) over 20 minutes followed by 20:80 A:B (v/v) for 5 minutes. A range of standards from 0 to 12.8 μg/ml of each ACE inhibitor were ran according to the same method and standard curve plotted (Figure 4.2). Peak area was compared to the standard curve to give concentration in the medium. The concentration of lisinopril eluted into the medium was much higher than initially thought, so a range of more concentrated lisinopril standards were prepared and plotted to give a fuller standard curve.

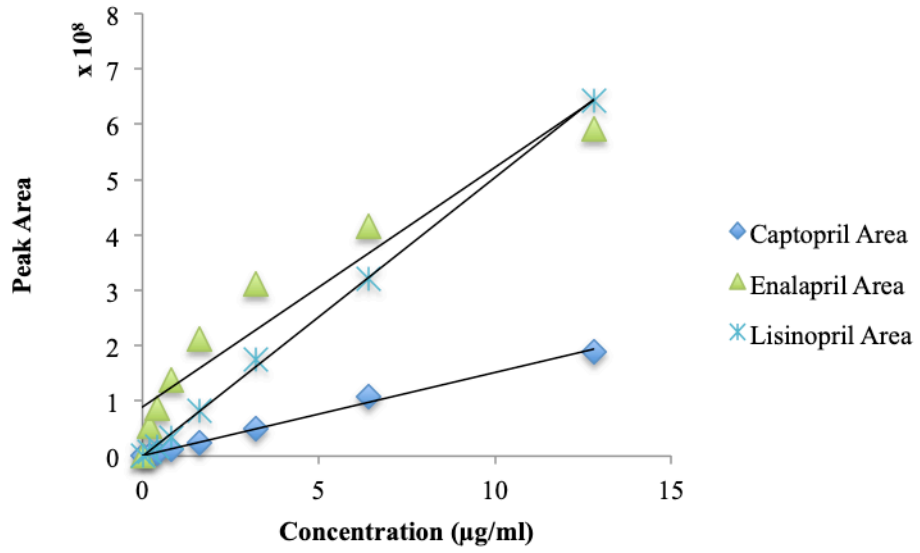


Figure 4.2: Standard curve of peak area vs concentration of captopril, enalapril and lisinopril; Measured by liquid chromatography-mass spectrometry.

4.3 Results

4.3.1 Mechanical testing of gels containing ACE inhibitors with and without fibroblasts

The scale of the experiment required it to be completed in 2 parts. Firstly, a set of control, captopril, enalapril or, lisinopril containing gels were tested, using the Bose 3200 (Figure 4.3). Secondly, control, acetyl cysteine and collagenase inhibitor containing gels were tested using the Bose 3100 (Figure 4.5). As explained in section 4.1.5.2 (Page 97), an irreparable fault meant the testing could not be completed with The Bose 3200. Although this was unavoidable, it was not ideal, as it was found that the two machines had not been calibrated with each other. As the control sample was prepared in exactly the same way and tested on each machine this was used to compare the results obtained from the two machines. Because the Bose 3200 was

broken, there was no way to test a non-biological sample, which would be identical in both machines so the control samples, which had been prepared in the same way, were used to normalise the results. The results presented in Figure 4.4 and Figure 4.6 are presented as a function of the control of each group so as the results can be compared between experiments.

When comparing the results of the control gels tested in each machine, the trends for each seeding condition were similar (Figure 4.3 (a) and Figure 4.5 (a)). Gels prepared without cells, and with cells seeded throughout the gel, became increasingly compliant over the 9 days in culture, whereas gels with cells on their surface increased in stiffness. As discussed in section 4.4.1 (Page 121), developing a confluent cell layer on surface seeded gels may limit the efflux of the fluid phase from the hydrogel. This is turn could lead to the stiffness of the hydrogel being overestimated, as the incompressible fluid is trapped in the network.

Captopril containing gels (Figure 4.4 (b)) all followed a similar pattern with all seeding types initially increasing in stiffness before beginning to decrease. Gels with no cells and top seeded cells decreased in stiffness from day 6, and the suspended cells in gels from day 3.

Enalapril containing gels (Figure 4.4 (c)) all exhibited a slightly different pattern. Stiffness of gels without cells decreased from 0.57 ± 0.02 to 0.12 ± 0.02 before increasing to 1.91 ± 0.3 then again decreasing to 0.7 ± 0.16. Gels with suspended cells consistently decreased from 1.04 ± 0.19 to 0.43 ± 0.07 and with surface seeded

cells decreased from 0.70 ± 0.001 to 0. 22 ± 0.006, plateaued then increased to 0.46 ±0.2.

Results for Lisinopril containing gels (Figure 4.4 (d)), both those without cells and with surface seeded cells, were similar to those with captopril (Figure 4.4 (b)), initially increasing in stiffness then decreasing from day 6. Gels with suspended cells showed a similar pattern to the enalapril containing gels with top seeded cells, decreasing in stiffness from day 0 to day 3, plateauing and then increasing in stiffness.

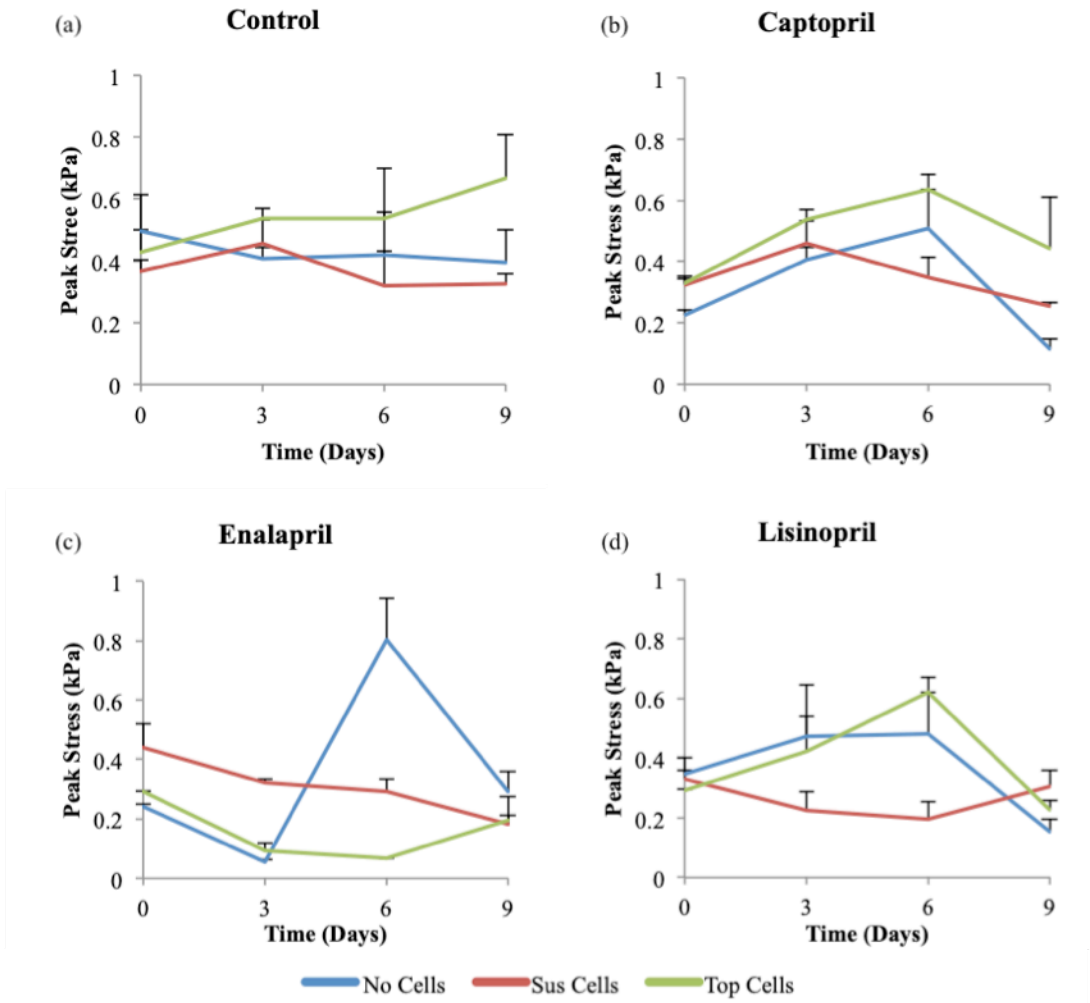


Figure 4.3: Peak stress of collagen gels against time of incubation up to 9 days after setting. Gels compressed by 5% strain containing no additive, captopril, enalapril or lisinopril. Gels prepared with cells on top, suspended throughout or without cells and tested with Bose 3200. n=3, mean + SEM.

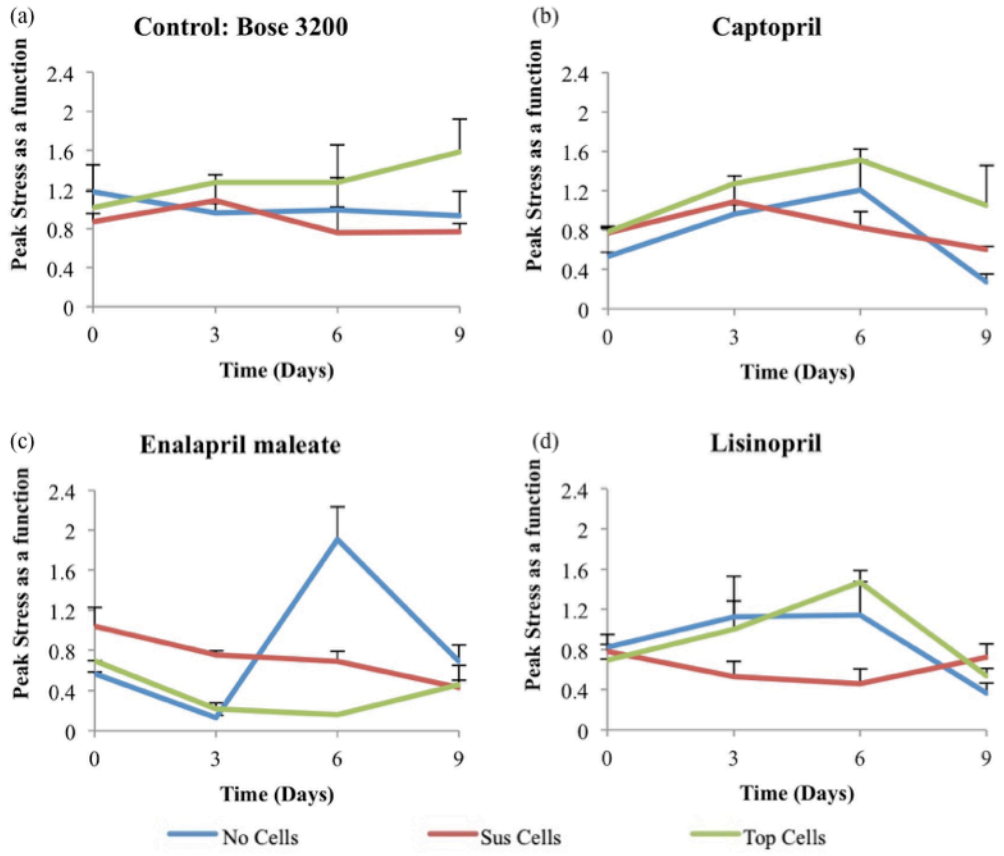


Figure 4.4: Peak stress of collagen gels normalised to the control against time for each gel. Gels compressed by 5% strain containing: (a) no additive, (b) captopril, (c) enalapril, or (d) lisinopril. Gels prepared with cells on top, suspended throughout or without cells and tested with Bose 3200. n=3, mean + SEM.

4.3.2 Mechanical testing of gels containing collagenase inhibitor and acetylcysteine

The control gels of the group tested on the Bose 3100 (Figure 4.5 (a)) show a similar trend to the control of the group tested on the Bose 3200 (Figure 4.3 (a)). Differences in stiffness between the control gels without cells and with surface-seeded and suspended cells are much more pronounced than those control gels tested with the Bose 3200 (Figure 4.4 (a) and Figure 4.6 (a)). Hydrogels containing acetyl cysteine (Figure 4.5 (c)) showed a similar trend to those containing enalapril, as well as the

control samples (Figure 4.3 (c) and (a) respectively). The behaviour of gels prepared with surface seeded cells closely follow the shape of the enalapril gel graph beginning with a stiffness of 1.05 ± 0.21 decreasing to 0.45 ± 0.11 at day 3 then increasing again to 1.07 ± 0.07. Acetyl cysteine containing gels with cells suspended in the matrix showed results similar to control gels with suspended cells in that they are stable then decrease, starting at 0.81 ± 0.10 at day 0 then decreasing from 0.85 ± 0.13 at day 6 to 0.49 ± 0.11 at day 9. The stiffness of acetylcysteine containing gels without cells however only begins to decrease at day 6 compared to the stiffness of the control gels, which begin to decrease at day 3. The collagenase inhibitor results are the most stable over the 9 days in culture. The graph of peak stiffness of collagenase inhibitor containing gels with surface seeded cells only differs between 1.31 and 1.51 and gels prepared without cells look very similar in shape to both Lisinopril and Captopril containing gels without cells increasing from 0.75 ± 0.15 at day 0 to 1.04 ± 0.26 at day 6 then decreasing to 0.65 ± 0.17 at day 9. Gels prepared with cells suspended in the matrix have a lower stiffness than the top seeded cells but also remain stable over the 9 days differing between 0.58 ± 0.14 and 0.37 ± 0.08.

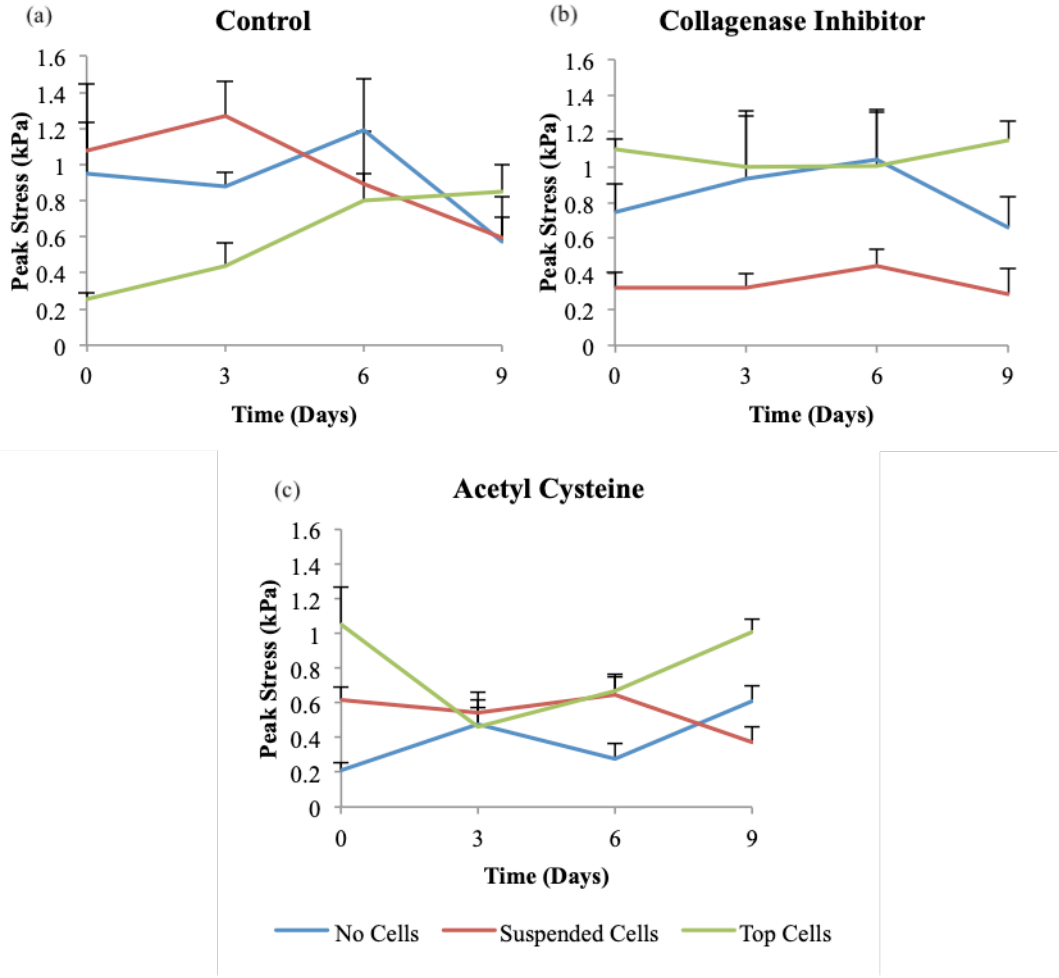


Figure 4.5: Peak stress of collagen gels against time of incubation up to 9 days after setting. Gels compressed by 5% strain containing: (a) no additive, (b) mammalian collagenase inhibitor or (c) acetyl cysteine. Gels prepared with cells on top, suspended throughout or without cells and tested with Bose 3100. n=3, mean + SEM.

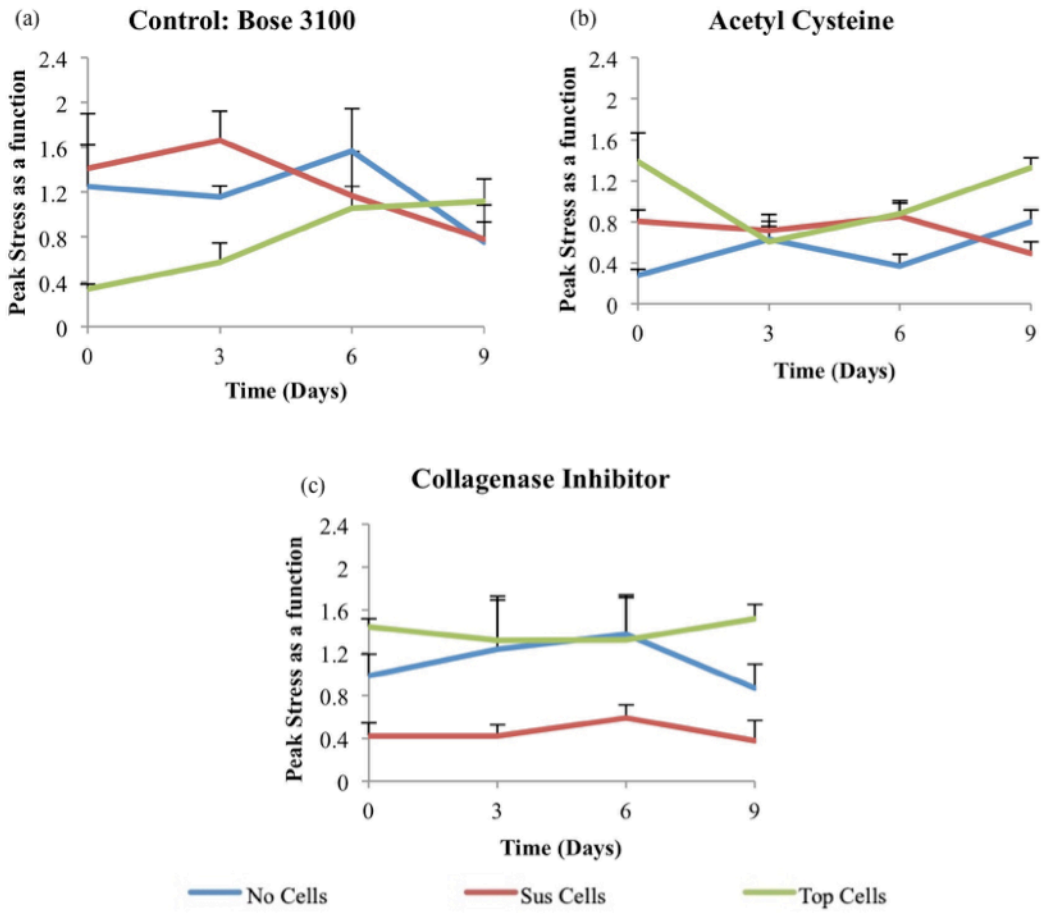


Figure 4.6: Peak stress of collagen gels as a function of the control against time for each gel. Gels compressed by 5% strain containing no additive, acetyl cysteine or collagenase inhibitor. Gels prepared with cells on top, suspended throughout or without cells and tested with Bose 3100. n=3, mean + SEM.

4.3.3 MTT of fibroblasts in collagen hydrogels containing ACE inhibitors, collagenase inhibitor or acetylcysteine

Cell viability was measured by an MTT assay. Collagen gels contained 1 mM of each ACE inhibitor and 3T3 fibroblasts were then either surface seeded, or cells suspended throughout the gel. Cells seeded on the surface of gels increased in number in all gels except in gels containing captopril. Absorbance was recorded at day 3, 6 and 9 and compared to a cell number standard curve. Each gel was seeded

initially with 57,000 cells (displayed by a solid black line on Figure 4.7 and Figure 4.8). Cells in all gels increased over the 9 days except those containing captopril and acetyl cysteine. A two-way ANOVA was conducted to compare time and drug and the interaction effect of time and drugs on cell number. All factors were significantly different at the 0.05 confidence interval. The 9-day results of each group were compared by one-way ANOVA. No significant difference was found between control, enalapril and lisinopril gels for suspended cells or top seeded cells for suspended or top seeded cells. A significant difference was found between the captopril gels and control, enalapril and lisinopril gels.

Control, enalapril and lisinopril gels prepared with surface seeded cells all increased from their initial seeding density of 57,000 cells at day 0 to 205,237 ± 5,550, 186,783 ± 6,150 and l 171,072 ± 2241 cells respectively (Figure 4.7 (a)). Captopril had a cytostatic effect on cells with cell number increasing slightly from day 0 to day 3 then remaining fairly stable over the 9 days. After 9 days in culture, there was a greater increase in cell number in Control, Enalapril and Lisinopril gels prepared with suspended cells, than in the equivalent gels prepared with surface-seeded cells (Figure 4.7 (b)).

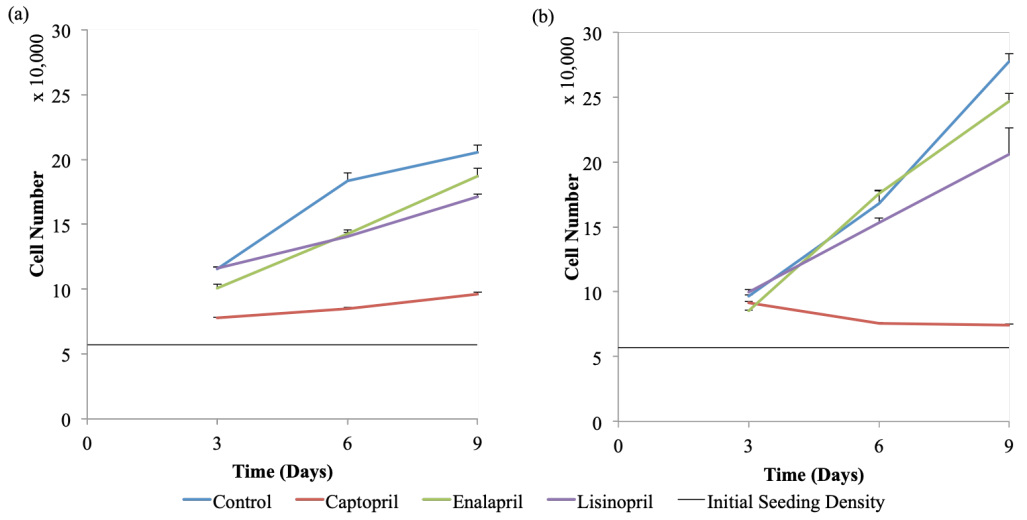


Figure 4.7: Number of cells measured by MTT against time. 3T3 fibroblasts (a) seeded on gels surface or (b) suspended throughout the gel. Gels contained no additive, captopril, enalapril or lisinopril. Initial seeding density of 57,000 cells indicated by line. n=4, mean + SEM.

The experiments were repeated with collagenase inhibitor and acetylcysteine in place of the ACE inhibitors (Figure 4.8) to attempt to elucidate a mechanism of action for the effects shown by the ACE inhibitor experiments. One-way ANOVA of the 9 day results showed no significant difference between any of the surface seeded gels but found a significant difference between all the suspended gels at the confidence interval 0.05. Surface seeded gels increased in number in all three gel preparations (Figure 4.8 (a)), increasing to 358,616 ± 10,907 in control gels, 344,513 ± 7,208 in collagenase inhibitor gels and 280,791 ±15,532 in acetylcysteine gels after 9 days. The number of suspended cells also increased in control and collagenase inhibitor gels over 9 days (Figure 4.8 (b)) to 201,957 ± 13,570 and 315,018 ± 19,605 respectively however cell numbers in suspended acetyl cysteine containing gels remained stable at about 70,00 cells over the 9 days.

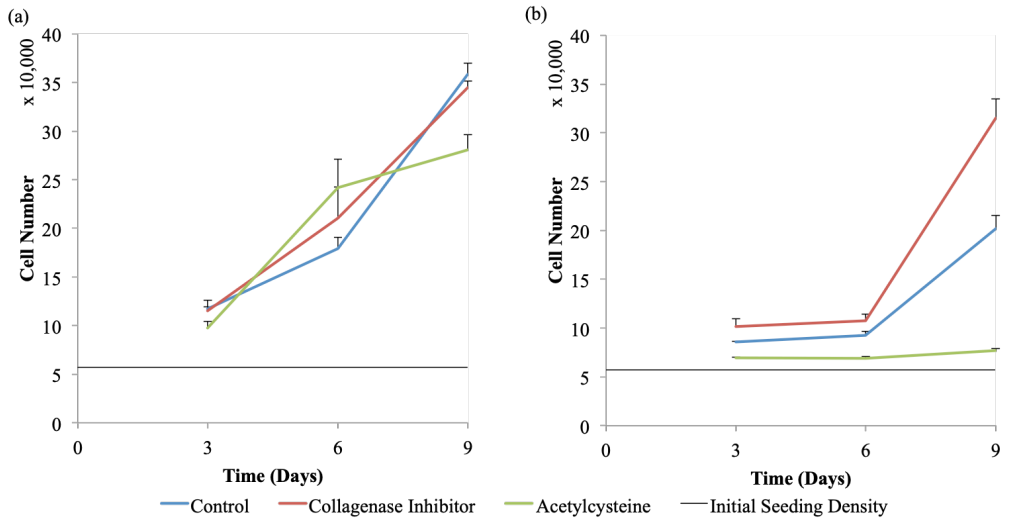


Figure 4.8: Number of cells measured by MTT against time. 3T3 fibroblasts (a) seeded on gels surface or (b) suspended throughout the gel. Gels contained no additive, collagenase inhibitor or acetyl cysteine. Initial seeding density of 57,000 cells indicated by line. n=4, mean + SEM.

4.3.4 Contraction of FPCLs containing ACE inhibitors

Contraction of hydrogels was detected by measuring area of gels over time (Figure 4.9). Gels were prepared with or without 3T3 fibroblasts and 1 mM ACE inhibitors and were left free-floating or attached to the sides of the plate for up to 9 days. None of the gels left attached to the plate changed in size. All gels had an initial area of 19.6 cm^2. Free-floating control gels without cells increased in area due to self-compression, while those with suspended cells initially increased in area to 23.17 cm^2 then contracted to 15.10 cm^2. Surface seeded cells contracted from day 0 to day 9 to 11.73 ± 0.32 cm^2. Gels prepared with captopril showed very little contraction, initially all free-floating gels expanded. Surface seeded gels began to contract a little after their initial expansion however only contracted to 19.27 ± 0.07 cm^2 over the 9 days. Enalapril supplemented gels without cells also increased in area. When prepared with suspended and surface seeded cells, enalapril gels increased in

area to day 3 then contracted to 9.7 ± 0.36 and 14 ± 0.59 cm^2 respectively. Lisinopril supplemented gels with surface seeded and suspended cells contracted from day 0 to day 9 consistently to 10.4 ± 0.15 cm^2 and 7.83 ± 0.32 cm^2 respectively. The lisinopril gels without cells also expanded. When compared by ANOVA at day 9, the area of control gels were significantly different to captopril, enalapril and lisinopril gels both with suspended and surface seeded cells ($p<0.05$).

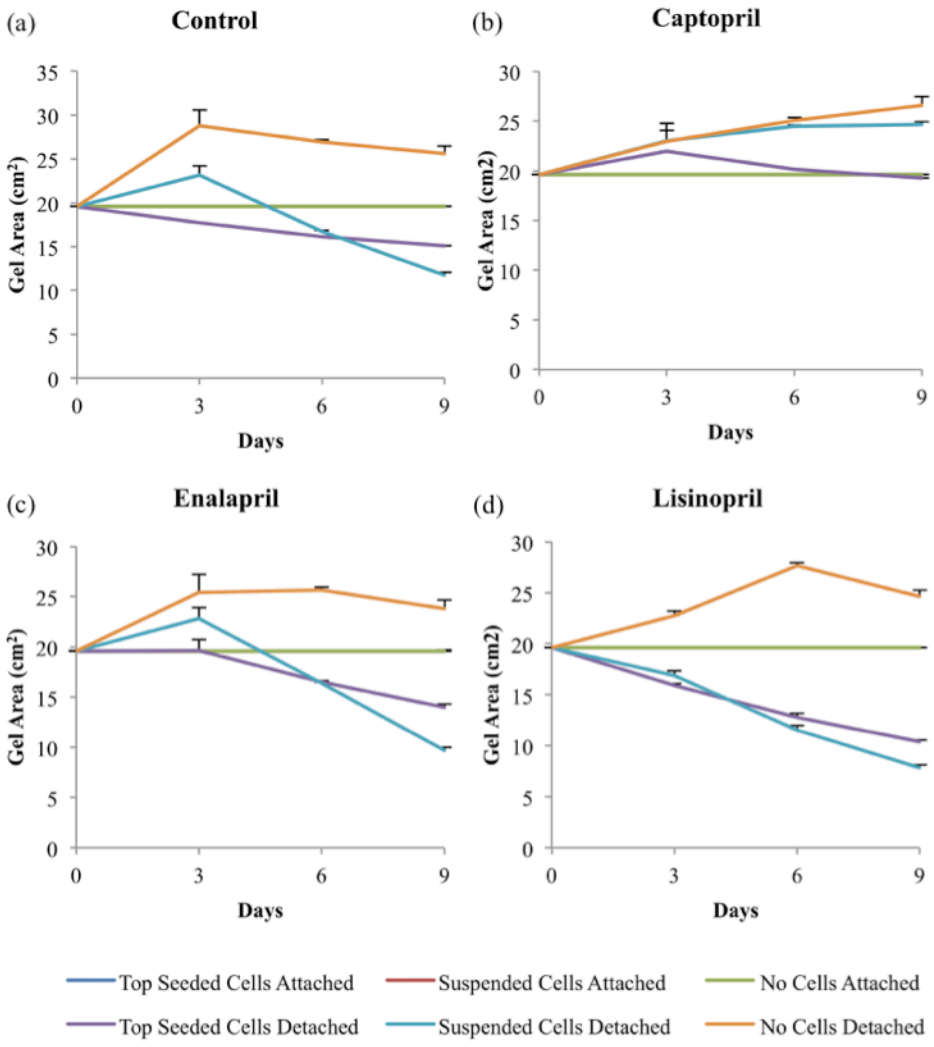


Figure 4.9: Area of FPCL measured against incubation time. Gels contained no cells or had 3T3 fibroblasts seeded on the surface or suspended throughout the gel. Gels were supplemented with (a) no additive, (b) captopril, (c) enalapril or (d) lisinopril prior to setting. Half the gels were detached from the tissue culture plate and moved to a larger dish, the other half were left attached to maintain the gels in tension. n=3, mean + SEM.

Weight of gels (Figure 4.10) decreased in all gels, with greater expansion or contraction correlating with greater weight loss. Gels without cells changed in weight less than their counterparts with cells.

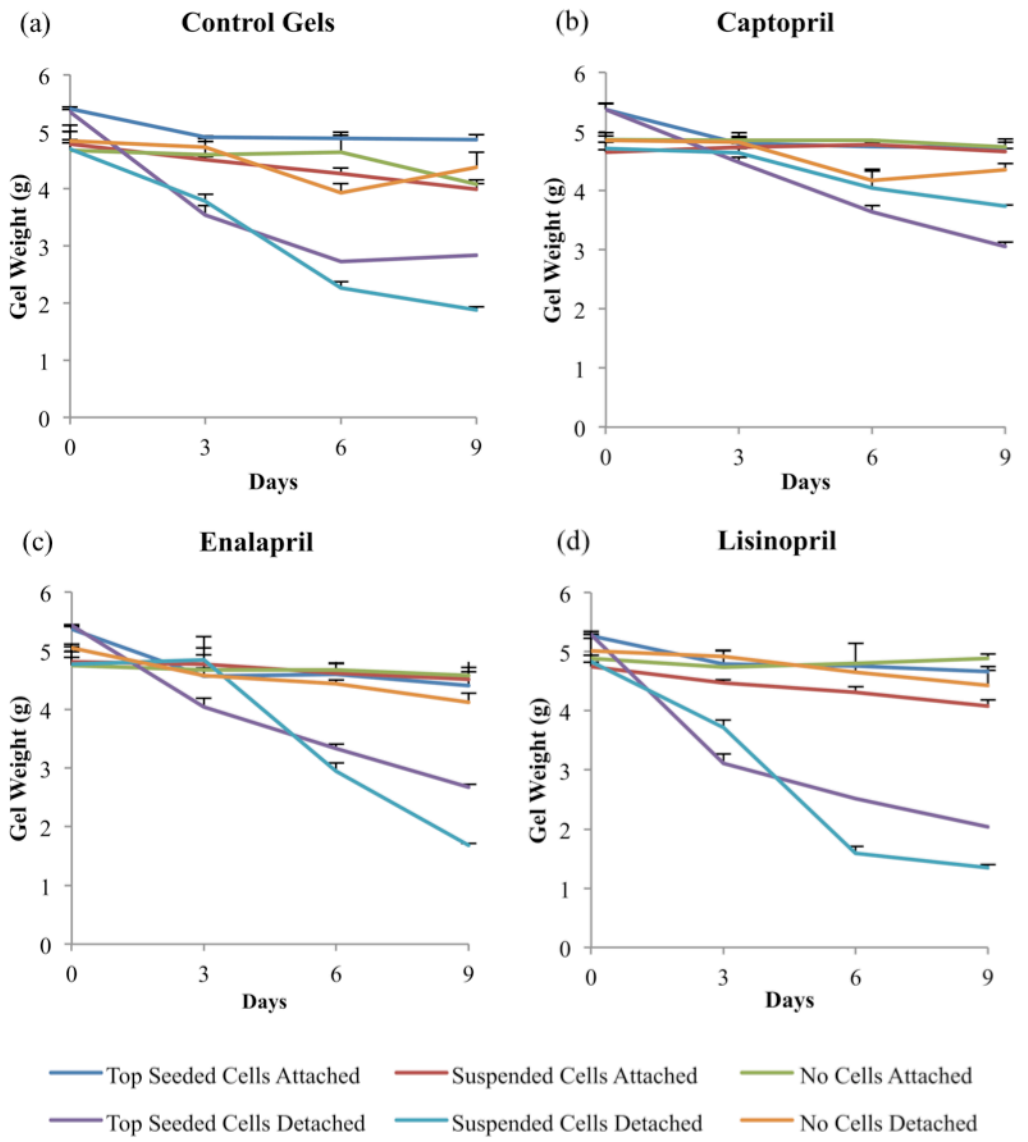


Figure 4.10: Weight of FPCL measured against incubation time. Gels contained no cells or had 3T3 fibroblasts seeded on the surface or suspended throughout the gel. Gels were supplemented with (a) no additive, (b) captopril, (c) enalapril or (d) lisinopril prior to setting. Half the gels were detached from the tissue culture plate and moved to a larger dish, the other half were left attached to maintain the gels in tension. n=3, mean + SEM.

4.3.5 Mass Spectroscopy of ACE inhibitor release from hydrogels

Mass spectroscopy was employed to measure elution of ACE inhibitors from collagen gels containing captopril, enalapril or lisinopril at a concentration of 1 mM (Figure 4.11). The collagen gels were kept at 37 °C, then, the medium removed from the collagen gels after 30 minutes, 1 hour, 6 hours, 1 day, 2 day or 3 days and diluted in acetonitrile at a ratio of 4:1 acetonitrile:medium sample. The samples were centrifuged at 132,00 RPM for 10 minutes then stored at -80 °C. The concentration of ACE inhibitor was measured in the media samples by LC-MS. The concentration of captopril was consistent over the 3 days, at a concentration of 0.025 mM. Enalapril elution into medium increased from a concentration of 0.11 mM after 30 minutes to 0.39 mM after 3 days. Lisinopril elution into medium increased from 0.23 mM after 30 minutes to 0.7 mM after 3 days.

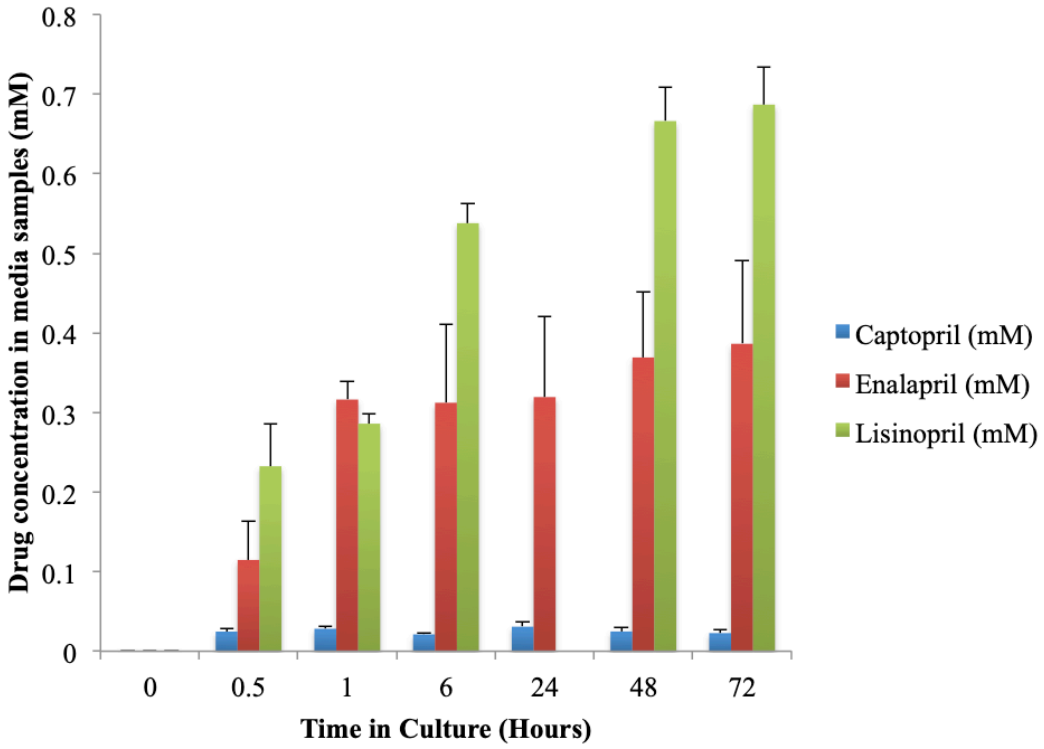


Figure 4.11: Concentration of ACE inhibitor eluted into media measured by liquid chromatography-mass spectroscopy. Collagen hydrogels supplemented with 1 mM of each ACE inhibitor. Initial media contained no ACE inhibitor, n=3, mean + SEM.

4.4 Discussion

Previous research showed inhibition of collagenase activity by captopril and enalapril (Busby, 2013). Zymography showed concentrations much higher than clinically relevant doses of captopril and enalapril were required for inhibition of collagenases. This work used a relatively high concentration of 1 mM of each ACE inhibitor; a concentration found by Busby to inhibit collagenase activity without affecting cell viability. Comparatively, therapeutic *in vivo* serum concentrations for

treatment of hypertension are very low. In pharmacokinetic studies, peak captopril concentration in the plasma was reached within an hour of dose delivery; a dose of 10 mg gave sera concentrations of 0.66 ± 0.07 μM (Duchin et al., 1982). Enalapril maleate is a pro-drug converted by de-esterification to its active form enalaprillat in the liver (Gomez et al., 1985). Enalapril is undetectable in plasma after 4 hours (MacFadyen et al., 1993), while enalaprillat gave peak serum concentrations of 89 ± 34 ng/ml after 4.3 ± 1.7 hours (Bendtsen and Henriksen, 1989). Lisinopril gave peak concentrations of 64 ± 16 ng/ml after 7.5 ± 1.5 h (Beermann, 1988; Bendtsen and Henriksen, 1989). Although high concentrations of the drugs were required to show inhibition of MMPs by zymography (Brower et al., 2007), Sorbi et al demonstrated inhibition of MMPs at clinically relevant concentrations of captopril (Sorbi et al., 1993). Sorbi and colleagues hypothesised that the mechanism of inhibition depended on the sulfhydryl group contained in captopril chelating Zn^{2+}. The inhibitory effect was reversed when zinc was added to cell medium in excess. Much greater concentrations of lisinopril were required to produce the same inhibitory effects, this was thought to be because lisinopril does not contain a sulfhydryl group.

Mechanical properties of cell scaffolds are critical in cell scaffold design. Cell viability, behaviour and phenotype can all be affected by stiffness of the cell scaffold. Adding ACE inhibitors to collagenous hydrogels was hypothesised to inhibit cellular degradation of the scaffold, thus improving the mechanical stiffness of the hydrogel. Experiments to include high concentrations of ACE inhibitors and fibroblasts were designed to measure the mechanical properties of hydrogels. Cell

number, contraction of collagen gels and elution of the inhibitors was also investigated. Both mechanical testing and MTT are destructive tests so two sets of gels were prepared at the same time and in the same way to test each parameter.

4.4.1 Mechanical testing of collagen gels containing ACE inhibitors, collagenase inhibitor and acetylcysteine

As discussed in chapter 3, mechanical testing by confined compression gave a quick, reproducible and accurate method of testing the mechanical properties of control gels. Initially, the mechanical testing results were combined with biphasic theory models to give estimations of Hydraulic permeability and Young's modulus. Unfortunately, relaxation of the gels led to poor fitting of the experimental results to the models available, this process was abandoned and peak stress was used as a simple measure to infer stiffness of the gel. The same method was employed for testing mechanical properties of collagen gels containing cells and/or ACE inhibitors.

Three different cell-seeding conditions were chosen for mechanical testing: without cells; with cells seeded on the surface of the collagen gels; and with cells seeded throughout the collagen gels. Collagen gels without cell seeding were used as a basal control; theoretically, without added cells, collagen degradation should be minimal. There are two main methods of seeding cells in collagen. Either, hydrogels are set, then surface seeded with cells, or cells are suspended in the collagen mixture prior to setting to give a gel with cells suspended throughout. Surface seeded collagen gels are commonly used as raft like structures with the collagen gel being pre-set then cells added at a later time. This is a very easy method for culturing fastidious cells,

which do not grow well on polystyrene culture-ware. Confluent cell layers in surface-seeded gels were found to overestimate the stiffness of the hydrogel when tested in compression. Gels were confined in a well of a 24 well-plate and compressed to a 10% strain by a porous indenter, forcing any fluid to leave the matrix through the porous indenter. When measuring the stiffness of surface-seeded collagen gels, the confluent cell layer can create a barrier to upward fluid flow. As the gels are a biphasic material, with a solid phase comprised of a network of collagen fibres and a fluid phase of the interstitial fluid, the mechanical deformation by the porous indenter is initially supported by the incompressible fluid phase then subsequently transferred to the deformable solid phase (Chandran and Barocas, 2004) as the fluid leaves the gel through the porous indenter. Confined compression of surface seeded collagen gels can therefore overestimate the stiffness of the gels as fluid cannot be expelled through the confluent cell layer; it is believed the surface seeded cell gels are measured as stiffer than the underlying gel. Collagen gels prepared with cells suspended throughout the substrate provided a more tissue-like material as cells were evenly dispersed throughout an ECM like material: collagen. It was found that the fibroblasts were able to proliferate in both conditions.

Gels prepared without ACE inhibitor showed fairly stable mechanical properties in all cell-seeding conditions over the 9 days (Figure 4.4(a) and Figure 4.6(a)). As expected, the stiffness of surface seeded gels increased with increasing cell numbers, i.e. confluent cells blocked fluid expulsion, increasing the measured stiffness. In fact, in control gels, and both captopril and lisinopril gels, gels with the top surface seeded with cells are consistently stiffest when compared to gels with uniformly suspended

cells and gels without cells (Figure 4.4). This is consistent with a confluent surface cell layer and consequent blocking of fluid expulsion increasing the stiffness of the structure. Interestingly, captopril and lisinopril which are the two orally active ACE inhibitors tested, had a very similar effect on matrix stiffness. Captopril and lisinopril gels prepared without cells increased in stiffness from day 0 to 6 then decreased by day 9. Gels prepared with surface seeded cells followed the same pattern although were slightly stiffer than control gels. Captopril gels with suspended cells followed the same pattern, although reaching highest stiffness at day 3 rather than day 6 whereas the stiffness of lisinopril gels with suspended cells stayed fairly constant from day 0 to 6 then began to increase in stiffness towards day 9 (Figure 4.4(b,d)). Enalapril gels with suspended cells and top seeded cells decreased in stiffness slightly from day 0 to day 9. Cell free enalapril gels varied from day to day however the overall effect from day 0 to 9 saw a slight increase in gel stiffness (Figure 4.4(c)).

Captopril and Lisinopril both seemed to have a stabilising effect on the gels, initially improving the mechanical stiffness then slowing the weakening of the gels (Figure 4.4(b,d)). Three methods of action were proposed. Initially, it was suggested that captopril could be having a mechanical stabilising effect on the collagen triple helix. As captopril is the smallest ACE inhibitor, with a molecular weight of 217.3 g/mol, its molecules should be able to fit inside the collagen triple helix and act as a physical scaffold. A physical mechanism was also suggested to explain the effect of the lisinopril and enalapril structures, although they are much larger molecules with molecular weights of 492.5 g/mol and 405.49 g/mol respectively. It was hypothesised that rather than these molecules stiffening the collagen triple helix itself

they could potentially stiffen the gel by blocking the pores of the matrix, preventing fluid leaving the structure, thus increasing the perceived gel stiffness in a similar way to a confluent top seeded cell layer. An alternative hypothesis was that the gel stiffness was increased by inhibition of collagenase MMPs leading to reduced degradation of the collagen scaffold. ACE inhibitors have been shown in the literature to inhibit activity of MMPs (Brower et al., 2007; Levick et al., 2007) especially of the collagenase family (Table 4.4; MMP-2, MMP-9, 72 kDa and 92 kDa collagenases) (Sorbi et al., 1993; Brower et al., 2007; Yamamoto and Takai, 2009). The third hypothesis was that the stiffening arose from a chemical effect, with the ACE inhibitors facilitating crosslinking between side-chains of the collagen molecules. To investigate which of these hypotheses was most likely, the experiment was repeated to ascertain the mode of action. The mechanical testing and cell viability experiments were repeated, however the ACE inhibitors were replaced with a collagenase inhibitor and acetylcysteine in supplementing the collagen hydrogels. The collagenase inhibitor was chosen as it was known to inhibit the collagenases, and to replicate the structure of enalapril and lisinopril, as it had a similar molecular weight of 448.5 g/mol. Acetylcysteine was chosen as a small molecule, with a molecular weight of only 163.2 g/mol and a sulfhydryl group to replicate the hypothesised mechanical effect of captopril.

Table 4.4: Inhibition of MMP-2 by ACE inhibitors measured by zymography, adapted from Brower et al. (2007). Mean ± standard deviation. Control arbitrarily set to 100% activity as measured by MMP-2 activity without inhibition by ACE inhibitors. [concentration of ACE inhibitor] Significant difference from: *control ($p<0.01$); ^low dose ACE inhibitor ($p<0.05$).

	Control (%)	Low Dose (%)	High Dose (%)
Captopril	[0 mM] 100 ± 4.6	[0.25 mM] 82.6 ± 7.7*	[2.5 mM] 48.6 ± 8.9*^
Lisinopril	[0 mM] 100 ± 5.6	[0.35 mM] 77.8 ± 9.0*	[3.5 mM] 47.6 ± 8.7*^
Quinapril	100 ± 2.9	[0.1 mM] 84.0 ± 6.8*	[1.5 mM] 63.9 + 6.0*^

The collagenase inhibitor increased the stiffness of gels prepared without cells and with top seeded gels, however, it decreased the stiffness of suspended cell gels at all time points. Stiffness of gels prepared with acetyl cysteine was reduced in all preparations compared to the control. This suggests that the stiffening of the gels observed by the addition of the ACE inhibitors is not caused by the mechanical imposition of molecules in the triple helix (captopril). The results observed in the samples prepared with collagenase inhibitor in the gels without cells were consistent with the hypothesis of blocking pores in the matrix (enalapril and lisinopril). It is possible that these effects, and in particular the observed weakening of the gels containing suspended cells by the collagenase inhibitor as against the observed stiffening of the cell free and top seeded gels, are connected with the diminished viability of the cells seeded in the hydrogels caused by the sulfhydryl ring of the captopril and acetylcysteine molecules (see section 4.4.2).

Three ACE inhibitors were chosen for this study, each of which had been shown to inhibit MMP activity (Brower et al., 2007; Levick et al., 2007; Jin et al., 2007). Captopril was chosen as a small molecule, which could realistically fit inside the collagen fibril, while lisinopril was chosen as a large drug which had been shown to inhibit MMP at low concentrations (Brower et al., 2007). Enalapril was chosen as a pro-drug to investigate whether it would have any affect on the scaffold without being metabolised. Both enalapril and captopril have also been shown to attenuate type-I collagen production, a side effect that has been exploited to treat cancer and limit fibrosis (Ihm et al., 1994; Molteni et al., 2003). Claridge et al. found ACE inhibitors to have the opposite effect and promote Type II collagen synthesis (Claridge et al., 2004). The effect of ACE inhibitors on hydrogels could therefore be multi-fold, with the attenuation of collagen formation balancing the reduction in collagen degradation. ACE inhibitors are known to also inhibit MMPs but sulfhydryl groups, which form part of the captopril molecule are known synthetic inhibitors of the wider class of zinc catalysed endopeptidases (Vincenti et al., 1994; Dunten et al., 2001; Isaksen and Fagerhol, 2001). ACE inhibitors can also have an antioxidant effect, which could affect cells in culture (Sorbi et al., 1993). Without further tests, the mechanisms by which ACE inhibitors are affecting the hydrogels cannot be ascertained with certainty.

4.4.2 Cell viability of fibroblasts in gels containing ACE inhibitors, collagenase inhibitor and acetylcysteine

Cell viability was measured by the MTT assay. MTT measures cell metabolism rather than cell viability directly. However, cell number can be inferred as generally increased cell number results in increased cell metabolism. Both seeding conditions

(suspended and surface seeded cells) resulted in significant increases in cell numbers over the 9 days of the experiments, with suspended cells in control gels increasing 5 fold compared to 3.5 fold when on the surface of the gels. Although there were still viable cells in captopril containing gels, there was a significant difference in cell number in gels containing captopril compared to control gels. The cell number remained constant in gels containing captopril over the 9-day incubation period. This suggests captopril had a cytostatic effect on cell growth. The effect was more pronounced in gels containing suspended cells, most likely because the dissolved drug surrounded the cells rather than having limited contact with the drug on the surface seeded gels. Results published in literature showed captopril had an anti-mitotic effect on lung fibroblasts in culture, which halted fibroblast proliferation (Nguyen et al., 1994; Molteni et al., 2003). Molteni and colleagues suggested the cytostatic effect could arise from the sulfhydryl group of captopril, as lisinopril, a non-sulfhydryl ACE inhibitor, did not produce the same effect. There was no significant difference in cell numbers between control, enalapril and lisinopril for each of the seeding conditions, as shown by statistical analysis. This was consistent with the observations in the literature. To investigate further whether the sulfhydryl group was at play in the cytostatic effect of captopril, an experiment with acetylcysteine was devised. Like captopril, acetylcysteine contains a sulfhydryl group. The MTT activity of gels containing acetylcysteine showed increased cell numbers in top seeded cells but no increase in suspended cells. This suggests that the sulfhydryl group does play a role in the cytostatic effect, although it may only have a very localised effect as surface seeded cells are less affected at the interface between the gel and the cell layer. Cell numbers on samples prepared with surface seeded

cells and collagenase inhibitors were not significantly different to the control gels, however, cell numbers were greater in samples prepared with collagenase inhibitor and containing suspended cells than the control gels. The collagenase inhibitor therefore seemed to have a positive effect on cell proliferation in cells suspended in the matrix. This is an unexpected result. It is generally believed that collagenases promote cell proliferation by breaking down the matrix and thereby assisting cells to migrate through the degraded ECM (Riley and Herman, 2005). The gels prepared with collagenase inhibitor and suspended cells were also most compliant when compared to control gels and those prepared with surface seeded gels. Whether increased cell numbers caused this compliance by degradation of the surrounding matrix, or increased compliance improved cell viability is unknown.

4.4.3 Contraction of FPCLs containing ACE inhibitors

Bell and colleagues developed a technique incorporating fibroblasts in free-floating collagen lattices, which has been widely used as a wound-healing model (Bell et al., 1979). To be an effective cell scaffold, fibroblasts must be able to proliferate, migrate and remodel their scaffold environment. Collagen gels containing fibroblasts were prepared containing ACE inhibitors. The contraction and weight of the gel was measured over 9 days to compare the negative control to ACE inhibitor treated gels. Both enalapril and lisinopril showed very similar contraction, and it compared well to the negative control gels. There was no contraction of collagen gels treated with captopril. As noted above captopril had a cytostatic effect on cell number when measured by MTT although the gel did still contain some metabolising cells. This suggests that the captopril treated cells have lost their ability to migrate and remodel the gel, as well as their ability to proliferate. Inhibition of ACE per se has no effect

on the extent of contraction; however, the sulfhydryl group of the captopril does appear to inhibit contraction.

4.4.4 Elution of captopril, enalapril and lisinopril from ACE inhibitor containing collagen gels

Elution of the ACE inhibitors was measured over 3 days by mass spectrometry. Gels were prepared containing 1 mM of each drug. The concentration of ACE inhibitor was measured in the media at each time point. Captopril was well retained, with a concentration of approximately 0.15 mM present in the media at each time point. The stability of captopril was queried, however, Pereira and Tam found captopril to be stable at 25 °C and 50 °C for 11.8 ± 1.2 and 3.6 ± 0.4 days respectively (Pereira and Tam, 1992) suggesting the drug would be stable for the 3 days at 37 °C in culture media. Elution of enalapril was much more rapid with a concentration 0.12 mM being measured in media after 30 minutes and reaching a concentration of 0.4 mM after 3 days. Lisinopril elution was even more dramatic with 0.25 mM measured in the media after 30 minutes and 0.7 mM present after 3 days. These results suggest that captopril could be entrapped within the collagen molecule, limiting the elution of the drug. Modelling by Prof. Simon Mackay suggested that it would be possible for the folded structure of the captopril drug to be present inside the helix of Type I collagen. Any effect that the lisinopril and enalapril are having on the collagen gel constructs are with very limited concentrations of the drug.

4.4.5 Potential mechanisms of action of ACE inhibitors

Three hypotheses were suggested and tested to elucidate the mechanisms responsible for the observed effects. The first hypotheses, a mechanical action by the ACE inhibitor molecules was proposed, with the drug molecules either blocking gel pores or stabilising the structure of the collagen molecules effectively by reducing the "springiness" of the collagen triple helix coiled spring like structure. The first potential effect was considered in particular in relation to the action of the larger lisinopril and enalapril molecules and the second effect was considered most likely to apply to the smaller captopril molecule. The experimental results, however, suggested these were the least likely hypotheses given the reduction in stiffness observed in the enalapril containing gels.

A second hypothesis was that the ACE inhibitors acted on the gels through a biological inhibitory effect, with the drugs inhibiting the activity of collagenase released by cells and so slowing the degradation of the ECM. As ACE is a zinc metalloproteinase (Turner, 2003; Riley and Herman, 2005), there is a distinct possibility that compounds which inhibit it may also inhibit other zinc metalloproteinases. ACE inhibitors have been widely postulated and reported to inhibit collagenases, part of MMP family (Sorbi et al., 1993; Sakata et al., 2004; Williams et al., 2005; Brower et al., 2007; Levick et al., 2007; Tanaka et al., 2007). This seemed the most likely hypothesis as the inhibitory effect on collagenases is so widely published. However, the results presented here alongside further SDS-PAGE studies proved inconclusive as to whether this was the cause of changes in stiffness of the test samples. Given the widely reported links between ACE inhibitors and

collagenase inhibition and the assumed consequential reduction in collagen degradation it is possible that the lack of significant observable effects on collagen hydrogel stiffness most likely results from occurrence of a balancing effect, possibly the inhibition of collagen formation.

The third, and final hypothesis was that the ACE inhibitors had a chemical effect, with the drug molecules reacting with the main collagen polymer or its side chains to cross link the polymer chains and consequently increase gel stiffness. There was no evidence of this mechanism.

4.4.6 Conclusions of ACE inhibitor studies

The results from these experiments proved inconclusive as to the effects of the ACE inhibitors on the mechanical properties of the collagen gels and the mechanisms for these effects. Most likely, measuring bulk stiffness of the gels was not sensitive enough to measure minor mechanical changes. Further testing to quantitatively measure collagenase inhibition and its effect on collagen degradation and collagen synthesis would help to elucidate a mechanism of action. Testing mechanical properties at the cellular level, possibly by using AFM or nano-indentation, could also prove useful, as mechanical changes are likely to be significant at the micro rather than macro level.

The results of experiments to test the effect of the different ACE inhibitors on cell viability were more conclusive, with captopril apparently inhibiting cell proliferation while enalapril and lisinopril had no harmful effect on cell proliferation. Therefore, further work on the mechanical effect should concentrate on those ACE inhibitors,

and additional further work to better understand the mechanisms by which the addition of the collagenase inhibitor increased cell proliferation should be undertaken.

Chapter 5

Controlled dehydration of collagen hydrogels

5 Controlled dehydration of collagen hydrogels

5.1 Introduction

5.1.1 Plastic compression

Collagen hydrogels are useful biomimetic tools for promoting cell viability in three-dimensional culture. Their main drawback for practical applications is their mechanical weakness; they cannot support their own weight when removed from culture medium. Plastic compression as a method of improving the mechanical properties of collagen hydrogels by controlled dehydration was developed by Robert Brown's group at University College London and proved effective for quickly improving the stiffness of collagen hydrogels (Brown et al., 2005). The system could be utilised for a variety of research applications, to provide 3-D cell culture matrices supporting viable cells. Most other methods to improve mechanical stability of collagen hydrogels such as chemical crosslinking (Table 1.2, Page 13) involve toxic chemicals, which limits integration of cells in the matrix and the potential for in vivo applications. Although cross-linked gels could be rinsed thoroughly and subsequently seeded with cells, this does not simulate a truly 3-D culture, rather a layer of collagen on which cells can be cultured in 2-D.

Brown's method of unconfined plastic compression prepared collagen hydrogels, with or without cells, in specialised casting plates. The hydrogels were then removed from their casting plates and placed on blotting paper. A glass plate was placed on top of the gel to ensure there was only one fluid leaving surface. Weights were then placed on the glass plate to further increase the force exerted on the gel. The water

was forced out of the bottom of the gel, into the blotting paper below, by gravity, leaving a thin layer of dense collagen matrix (Figure 5.1 (a)). This method proved successful in maintaining approximately 80% cell viability within the matrix immediately after compression (Brown et al., 2005).To improve the stiffness of the scaffolds further, the compressed hydrogels were rolled and compressed again (Figure 5.1 (b)), to create a multi-layered structure (Brown et al., 2005; Abou Neel et al., 2006). This resulted in layered collagen scaffolds; however cells were not viable after this subsequent compression (Abou Neel et al., 2006). The method was further adapted by using capillary action instead of gravity alone. Dense plungers of tightly rolled filter paper were prepared and used to remove water from the hydrogels by capillary action. A specially designed rolling machine was produced to create the compacted rolls of filter paper (Alekseeva et al., 2011). This method had the advantage that gels could be maintained in any round tissue culture plate. The success of this method led to its commercialisation by Lonza, who created the raft system. This made the dehydration of 3D collagen scaffolds much more accessible, however it came at a cost of approximately £104 for a single 24 well plate and limited the system to compression of hydrogels prepared in wells of a 24 or 96 well plate. The aim of the current work was to create a new system of plastic compression of collagen hydrogels, which could be readily scaled, would support viable cells within the matrix itself, and would require no further equipment other than that available in a normal cell culture laboratory. Another aim was to create a more affordable method than that of the Lonza raft system.

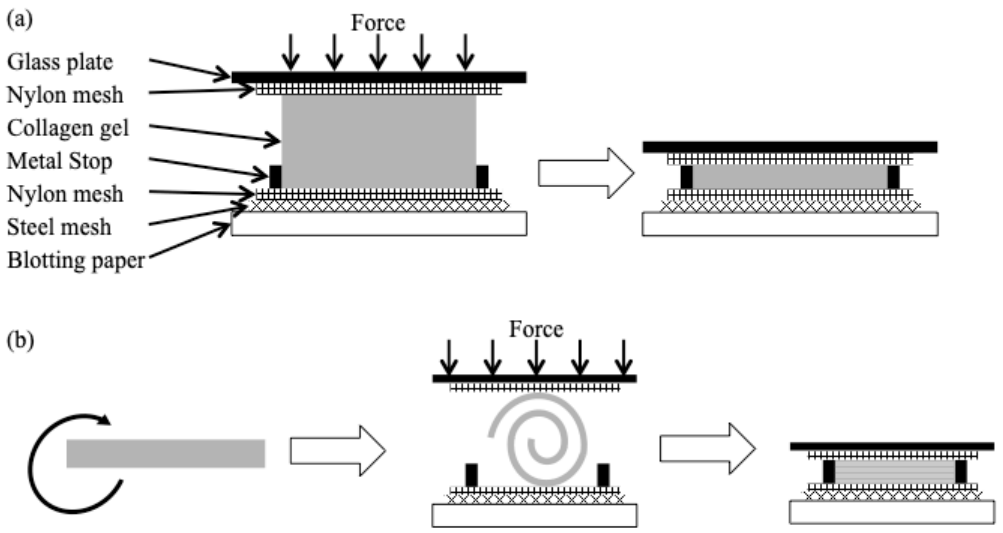


Figure 5.1: (a) Method of plastic compression described by Brown et al. (2005) in which fluid is forced from a collagen gel by forcing water from the hydrogel by gravity into blotting paper. (b) dehydrated hydrogel from (a) further compressed by rolling along the short axis and repeating the dehydration process to give multi-layered collagen specimens.

5.1.2 Super-absorber

Super-absorbers are polymers with a high affinity for water. These chemicals have a wide range of applications, from personal hygiene products to food and construction. There are a variety of chemistries available, making them highly tuneable for the desired purpose. To enhance the plastic compression of the gel by the removal of water, we hypothesised that a layer of super absorber could be used in place of blotting paper to increase the removal of water, and create a more controlled environment. For this application, a non-toxic super-absorber was required to ensure the hydrogels remained biocompatible with implanted cells. Therefore, if the approach was successful, the work could be later translated into devices for medical treatment in the human body. A super-absorber that formed a solid structure when wetted was also desirable so that it could be easily removed from the hydrogel

structure after the dehydration process was complete. Many super absorbers were investigated before sodium polyacrylate (SP) was chosen. SP is currently used in cleaning applications and as artificial snow. It is also used in a range of personal hygiene products such as babies' nappies and cosmetic creams as well as edible products such as sugar. It is approved for use in food for human consumption by the FDA, and for use in a range of cosmetic and personal hygiene applications by the Japanese Standards for cosmetic ingredients and the cosmetic ingredient review (Fiume, 2002) and deemed generally safe. The wide range of applications currently making use of SP make it readily available and very affordable.

5.1.3 Mechanical testing of collagen gels in tension

There have been many methods employed for mechanical testing of collagen hydrogels over the years. These methods range from rheology, measuring bulk mechanical properties at the macro scale, to atomic force microscopy, which measured stiffness of individual collagen fibrils at the micro scale. Cells behave very differently on scaffolds of different stiffness (Caliari and Burdick, 2016), and it is therefore an important factor to consider early during scaffold design. Replicating properties of the *in vivo* tissue, that the scaffold is intended to mimic, ensures that cells *in vitro* behave appropriately. Collagen hydrogels are a biphasic material made up of a solid phase (network of collagen fibrils) and a fluid phase (entrapped water). The high degree of hydration makes collagen hydrogels inherently weak thus difficult to test mechanically, particularly in tension. The physical properties of the material can give very different mechanical results, depending on the method of mechanical testing chosen. For example, a brittle material may have a high compressive strength but be very weak in tension, while a ductile material generally

has similar strength in compression and tension. It is, therefore, important to choose a method of testing that reflects the mechanical loading intended for the scaffold. As the dehydrated hydrogels in this work are most likely to be used in tension in their final applications, most commonly tissue engineering of skin or other tissues, a tensile mechanical testing method was chosen. The method was adapted from that developed by Saddiq and colleagues (2009), allowing testing of hydrated and dehydrated collagen gels in the same manner. As there is massive variation in stiffness of tissues and scaffolds reported in the literature, it was important to choose a measurement method that we could use to accurately compare the stiffness of different collagen samples confidently. We proposed to introduce several ECM components into the gels to optimise their properties. To this end, the GAG, HA, and ascorbic acid were chosen as detailed below.

5.1.4 Glycosaminoglycans

Glycosaminoglycans (GAGs) or mucopolysaccharides are an important family of (mostly) highly sulphated long un-branched polysaccharides present in the extracellular matrix (ECM). There are 4 groups of GAGs: heparan sulphate (HS); chondroitin sulphate (CS); keratan sulphate (KS); and hyaluronic acid (HA) based compounds. Apart from KS, all GAGs are made up of repeating units of an uronic acid (either glucuronic acid or iduronic acid) and an amino sugar (either N-acetylglucosamine or N-acetylgalactosamine). GAGs are heterogenous as unlike proteins or nucleic acids, their production is not ‘template driven’ (Scott and Panitch, 2013). Instead, production is dynamically controlled by the processing enzymes present (Raman et al., 2005). All GAGs are highly polar, allowing them to attract water, while also being very viscose and relatively incompressible.

HA is a simple polysaccharide and the only non-sulphated GAG. It is synthesised by hyaluronan synthases, which are integral in the cellular membrane and have transmembrane domains (Laurent and Fraser, 1986; Litwiniuk et al., 2016). Hyaluronan synthases both synthesise and translocate HA across the cell membrane to the extracellular matrix (Weigel and DeAngelis, 2007; Siiskonen et al., 2015; Köwitsch et al., 2018). HA is made up of alternating β-D-glucuronate and *N*-acetyl-β-D-glucosamine sugars (Litwiniuk et al., 2016) and has a very large MW normally ranging from 1-8 MDa (Cowman et al., 2015); although it can also be synthesised as a low MW polymer. High and low MW HA have different properties: high MW HA has immunosuppressive and anti-inflammatory properties while low MW HA initiates a pro-inflammatory response (Rayahin et al., 2015; Litwiniuk et al., 2016; Köwitsch et al., 2018). Generally, in healthy skin, the MW of HA is in the region of 4-6 kDa although low MW HA can be found in healing wounds (Cowman et al., 2015). The extensive hydrogen bonding gives HA a flexible 'worm-like' structure (Hart and Copeland, 2010), which allows the molecule to fill gaps in the extracellular matrix.

After collagen, HA is one of the most abundant molecules in the extracellular matrix. Human skin contains about 0.4 - 0.5 mg/g hyaluronic acid (wet weight), mostly in the dermis (Anderegg et al., 2014; Lee et al., 2016). HA has an extremely fast turnover rate *in vivo*, with a half life of only 1 day (Fraser and Laurent, 1989; Lee et al., 2016). It is constantly degraded by reactive oxygen and nitrogen species, hyaluronidases and MMPs meaning the ECM is constantly remodelling itself. The

polar nature of the hyaluronic acid molecule gives a very hydrophilic molecule, which can absorb up to one thousand times its own volume in water (Khunmanee et al., 2017). HA has important roles in lubricating and cushioning (Goa and Benfield, 1994) and maintaining the hydration of the skin (Juhlin, 1997; Papakonstantinou et al., 2012). It also has important roles in cell proliferation and migration although the mechanisms for this are not fully understood.

The main receptor for HA is CD44, which is present on the surface of nearly all human cells (Misra et al., 2015). The interaction of CD44 and HA is an essential part of the wound healing process as it signals fibroblasts from surrounding tissues to migrate into the wound site and activate the wound healing process (Litwiniuk et al., 2016). Over the years, HA has been extensively used in dermal fillers (Gold, 2007; Anderegg et al., 2014) and tissue scaffolds (Liu et al., 2004; Shah et al., 2008; Numata and Kaplan, 2011; Wang et al., 2013) as it promotes skin hydration and cell viability in the scaffold. It is an excellent molecule for tissue engineering applications because of its physicochemical properties as well as being biocompatible, biodegradable and non-antigenic.

This work was focussed on producing a skin-like tissue scaffold. HA was chosen for inclusion in the study as incorporating macromolecules in collagen scaffolds is important to better mimic native properties (Jia and Kiick, 2009). HA is an excellent choice for use in skin scaffolds as it is one of the most abundant molecules in skin after collagen. It provides viscoelasticity to the scaffold (Köwitsch et al., 2018), improving its mechanical properties. Viscoelasticity is an important parameter to

skin as it prevents injury by allowing skin to stretch and move without tearing (Everett and Sommers, 2013). HA also helps to maintain hydration in the scaffold. Healthy skin functions as a barrier to water loss and protection from heat, injury and infection (Sriram et al., 2015). HA provides a network to trap water and thus maintain bound water in the scaffold. Bound water molecules will not be lost from the scaffold by evaporation in the same way as free water is. The addition of HA will hopefully help to overcome the current problem of scaffolds quickly drying out when exposed to air. Our method of controlled dehydration of highly hydrated collagen hydrogels will prove useful in physically entrapping HA in a tight network of collagen and cells (Köwitsch et al., 2018). This method should also slow the degradation of HA. Maintaining hydration in a dehydrated scaffold may sound counterintuitive, however, this method should produce a cell-supporting scaffold, containing a high collagen concentration, which will maintain water content when exposed to air. HA also plays a cytoprotective role for cells, by attaching to cells like a coat and providing protection from reactive oxygen and nitrogen species, as well as promoting cell proliferation (Köwitsch et al., 2018).

5.1.5 Ascorbic acid

Ascorbic Acid or vitamin C (AA) plays an important role in skin health. It has several roles including: promoting collagen synthesis (Geesin et al., 1993), playing an antioxidant role and promoting cell proliferation (Pullar et al., 2017). AA is an essential co-factor in the collagen synthesis pathway; collagen cannot be produced without it. It is also a promoter of type I and III collagen production by fibroblasts (Tajima and Pinnell, 1996). Boyce and colleagues also found that AA played an important role in regulating keratinocyte culture as well as limiting cell contraction

after dermal grafting (2002). This is useful in limiting formation of scarring, one of the major causes of disability after severe burns. In this project, supplementing collagen scaffolds with AA was chosen to improve cell viability and increase the content of newly synthesised collagen in the matrix. It also had the added benefit of reducing free radicals, which in turn would benefit cells within the matrix.

5.1.6 Plastic compressed hydrogels for dermal scaffolds

To characterise the collagen hydrogels for use as dermal scaffolds, two parameters were measured: cell viability and collagen synthesis. It is an essential feature of a cell scaffold that cells are viable and proliferating. MTT, a colorimetric assay that quantifies cell metabolism products was chosen to measure cell viability. It is important, especially for *in vivo* applications, that the implanted collagen scaffold can be degraded at a suitable rate for de novo synthesis to replace the scaffold. A Pro-collagen I ELISA measured newly synthesised collagen in spent cell culture media. Pro-collagen I is the precursor to Type I collagen. It is subsequently hydroxylated to produce tropocollagen, therefore it is an excellent way to measure collagen synthesis while still able to distinguish it from the collagen originally in the collagen scaffold.

Quantifying collagen degradation was considered. Three different methods were explored: radiolabelling, ninhydrin assay or by monitoring production of a biomarker. Radiolabelling would have been a useful method of differentiating between newly synthesised collagen and degradation products from the original scaffold. Without extensive equipment and paperwork, this was not an available

technique. A ninhydrin assay has been used in the past to measure collagen degradation products, by quantifying free amines. This was not conducive to using with scaffolds containing embedded cells. The cells themselves require cell culture medium to survive, and produce free amines as part of their cell metabolism. It would be impossible to differentiate degradation products from those produced as a by-product from cell culture thereby masking any degradation of the scaffold itself. Biomarkers such as C1M, a MMP-1 generated collagen fragment, have been suggested to have clinical relevance in measuring collagen degradation in inflammation, osteoarthritis and other conditions (Henriksen and Karsdal, 2019). With more time, methods to measure such biomarkers could have been developed to quantify degradation products from the collagen scaffold. This is an obvious avenue for further research. Ideally, scaffolds could be engineered to degrade at the same rate as new collagen is synthesised, so cells within the scaffold can maintain the scaffold.

5.2 Methods

5.2.1 Materials

All materials, unless otherwise stated, were purchased from Sigma Aldrich, UK.

5.2.2 Controlled dehydration method

A method of controlled dehydration was devised using filter paper, cell strainer mesh and the super-absorber, sodium polyacrylate (SP). ASRT collagen gels were prepared in the appropriate pre-weighed tissue culture dishes as in section 2.3.2, Briefly, 0.3% gels prepared both with and without cells, were allowed to set for 2

hours in a 37 °C incubator. The gels were weighed before adding complete DMEM to allow full polymerisation. Gels were then returned to the incubator for 30 minutes before the DMEM was removed. For the dehydration, a section of cell strainer mesh was placed on the gel surface followed by a sheet of filter paper (Whatman Grade 1 qualitative filter paper), which initiated the dehydration. SP was placed on top of the filter paper, generally 10% w/v of the gel volume (i.e. 10 ml collagen gel dehydrated with 1 g SP), for a set amount of time. Various percentages of SP were tested, 10% w/v was found to give an effective dehydration whilst forming a gel of SP. Less SP affected the dehydration, while more SP was more effective but left excess SP unabsorbed. The SP, filter paper and mesh were removed and the resulting gel weighed again. The final weight of the gel was used to calculate the increased concentration of collagen and the percentage of the initial weight lost through dehydration.

5.2.3 Mechanical testing in tension

For mechanical testing in tension, gels were prepared as in section 5.2.2 in specially designed tissue culture plates (Saddiq et al., 2009). The casting chamber consisted of a lower plate with rubber gasket and upper plate containing a cut out casting chamber (Figure 2.3). These were sterilised by spraying with 70% propanol, and were allowed to dry in a laminar flow hood before being subjected to 30 minutes of UV light per side. To ensure sterility, the casting chamber was then placed into a 90 mm petri dish after assembling the upper and lower plates with 6 nylon screws.

Initially, samples comprising 8 ml of collagen solution were pipetted into the casting plate, and sections of nylon mesh added (Figure 2.4) before setting for 2 hours in the

incubator. The set gel was weighed, then complete DMEM added to complete polymerisation. For dehydrated gels (as in section 5.2.2): mesh, filter paper and SP were placed on the gel surface in the casting plate for a set time. The dehydration materials were removed, and the gel re-weighed.

All gels were maintained in their casting plates in a 37 °C incubator for up to 3 days before mechanical testing. Upon testing, the casting plates were separated and the lower plate placed into a bath of PBS (pH 7.4). The PBS bath was used to float the gel from the lower casting plate into a stainless steel spoon to maintain hydration and reduce the possibility of mechanical damage. The lower casting plate was removed from the bath and replaced with the lower half of the mechanical testing rig (lower grips and stage; Figure 2.5). The spoon containing the hydrogel was returned to the bath and the hydrogel floated into place on the testing rig. The upper grips were fixed and tightened on the rig, which was then removed from the PBS bath. As in Figure 5.2, the testing rig was attached to the Bose testing machine, then the stage removed. Digital Vernier calipers were used to measure the thickness of the gel when upright before subjecting the gel to an extension of 4 mm at a rate of 10 mm/minute (Figure 5.2).

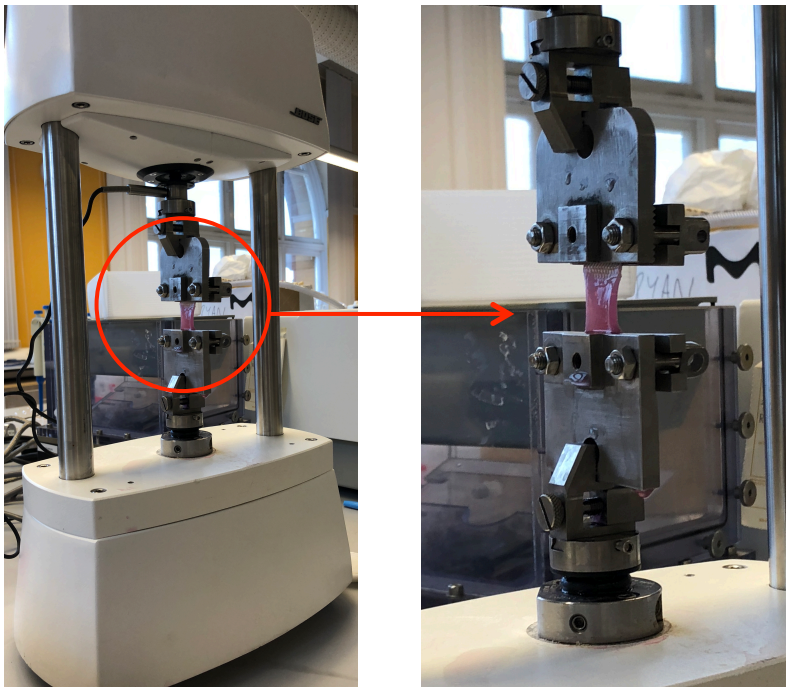


Figure 5.2: Left: Hydrogel during tensile mechanical testing with testing rig and Bose 3100. Right: Magnification of sample in testing rig with stage removed leaving grips at each end of the gel.

Most of the gels prepared using this method became separated from their embedded mesh prior to testing, because the mesh floated through the gel during setting. The method was improved to overcome this. 8 ml of collagen solution was set in the casting plate as before but allowed to set for 1 h 30 m before adding the nylon mesh. A further 2 ml of collagen solution was set on top of the gel and mesh for 30 minutes. This formed a sandwich of the nylon mesh within the collagen (Figure 5.4). After setting in the incubator, complete DMEM was added to complete polymerisation. Dehydration steps were then followed as in section 5.2.2 before adding complete DMEM and testing as before.

Load was recorded with Wintest software during the controlled displacement. Stress (Equation (i)) and strain (Equation (ii)) were then calculated and stress strain curve plotted (Figure 5.3). The initial non-linear plastic deformation was discounted and a gradient calculated from the linear section of the graph, giving a value for Young's modulus.

(i) $\sigma = \frac{F}{A}$

(ii) $\varepsilon = \frac{l - l_o}{l_o}$

where (i) σ=*stress, F=force, A=area*

(ii) ε=*strain, l=stretched length,* l_o=*original length*

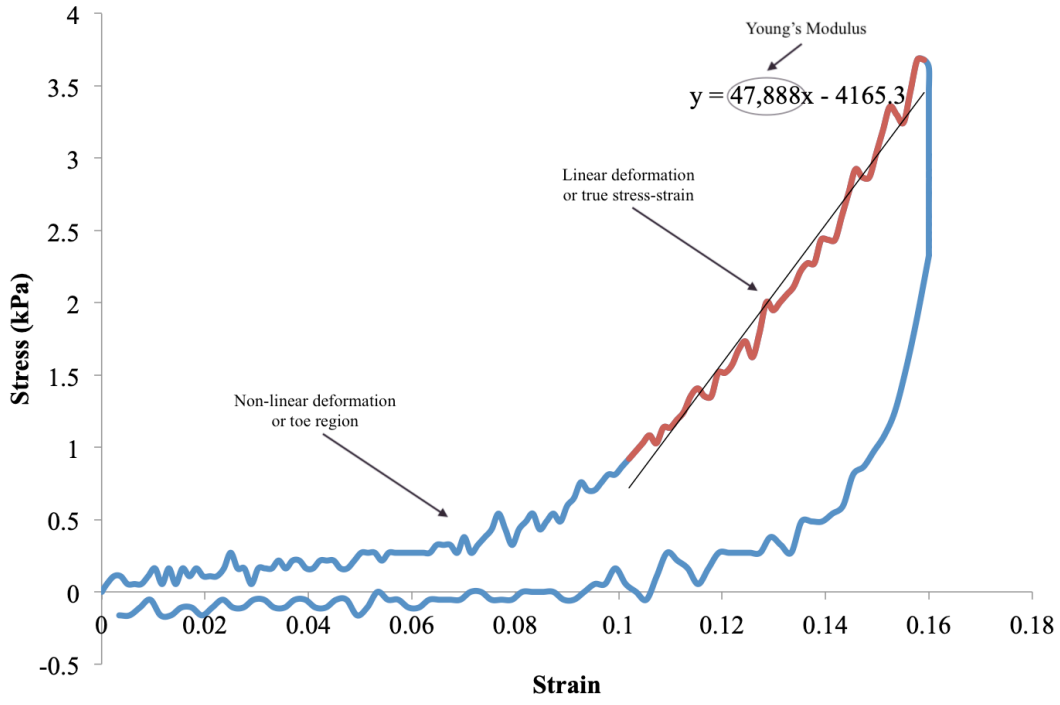


Figure 5.3: Stress strain plot of collagen hydrogels tested in tension outlining linear and non-linear areas of deformation and the gradient of the linear section of the graph, or the Young's modulus of the material.

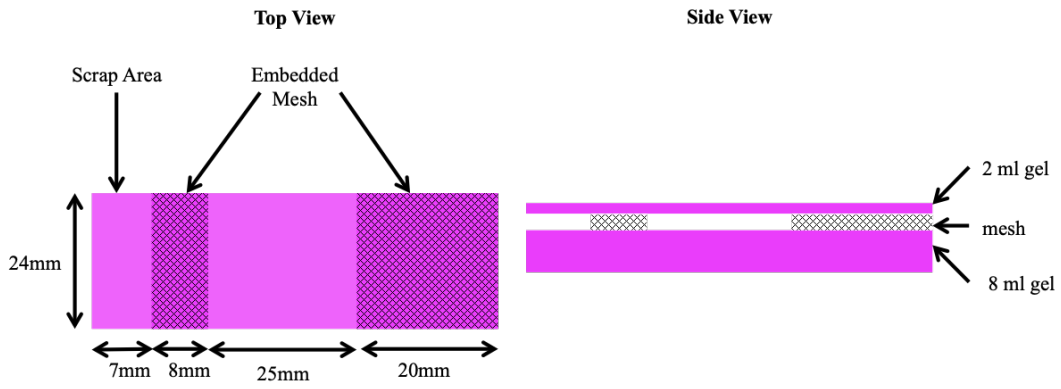


Figure 5.4: Measurements of embedded mesh in collagen hydrogel for testing mechanical properties in tension.

5.2.4 Addition of ascorbic acid and glycosaminoglycans

Samples of hydrogels were supplemented with hyaluronic acid (HA), ascorbic acid (AA) and both HA and AA.

HA was added to the relevant hydrogels prior to setting. A solution of 0.3% HA was prepared in sterile deionised water and mixed thoroughly until it had completely dissolved. A solution of 0.3% collagen was prepared as in section 2.3.2; the volume of acetic acid was adjusted to incorporate 10% of the final volume of the HA solution, giving a final concentration of 0.3% collagen and 0.03% HA. The collagen and HA solution was swirled to mix, and the pH adjusted to 8-8.5 with the drop-wise addition of 1 M NaOH, until a pink-purple colour was achieved.

The hydrogels were also supplemented with ascorbic acid (AA) at a concentration of 100 μM (Appendix: Figure i.iv) The AA was added to the media immediately before adding to the hydrogels, to minimise any breakdown and maximise the concentration available to the cells.

5.2.5 Measuring collagen synthesis

Newly synthesised collagen was measured in used cell culture media with a pro-collagen ELISA kit (AbCam, UK). The ELISA method was completed according to the manufacturer's instructions. Briefly, a sample of the medium was diluted by a factor of 1 in 40 in sample diluent. Standards were prepared with a concentration of 0 to 2000 human pro-collagen I pg/ml. To each well of the pre-coated ELISA wells, 50 µl of sample and 50 µl of antibody cocktail were added. The plate was incubated at room temperature for 1 hour on the plate shaker. Each well was then washed 3 times with wash buffer solution and 100 µl of colour development solution added for 10 minutes. Stop solution (100 µl) was added to each well and absorbance was measured with a plate reader at 450 nm.

5.2.6 Statistical analysis

Details of the statistical analysis adopted in each experiment are described alongside the results. Admittedly, many of the n numbers are low for experimental reasons, further described in the text, and whilst statistical analyses are presented, they should be treated with some caution. Where significant differences have been found ($p < 0.05$) these would be indicative of a big effect size; however, it must be noted that random experimental error could have a disproportionate effect in experimental tests with low n. In addition, the corresponding low statistical power of the analyses probably guarantees that some significant differences have been missed. Due to the above comments, additional statistical analysis and interpretation are provided in an Appendix and not provided in the main body.

5.3 Results

5.3.1 Dehydration of collagen gels with and without cells

To validate the method of dehydration and to observe how the dehydration proceeded with time, gels without cells were prepared from reconstituted type-I collagen from rat-tail tendon. Gels were prepared without embedded cells and dehydrated with 10% (w/w) SP for 5, 10, 20, 30, 45 or 60 minutes as described in section 2.3.2 (Figure 5.5). The gel weight decreased by 96% in the first 10 minutes of dehydration, equivalent to a 25 fold increase in collagen concentration. By 60 minutes, the gel had been reduced to 2.3 % of its original weight. As the collagen fibrils in the gel are not absorbed by the super-absorber, which was verified by staining the super absorber with Sirius red, the concentration of collagen in the gel after 60 minutes was determined as 12.8%. When compared by one-way ANOVA followed by a post-hoc Tukey analysis, there was a significant difference ($p<0.05$) between the concentration of collagen in the hydrated hydrogels and the concentration in the dehydrated gels at all the time points in the dehydration tests (Appendix: Table i.i). There was, however, no significant difference in the concentration of collagen between the dehydrated gels at each time point, suggesting strongly that the vast majority of the dehydration occurred within the first 10 minutes.

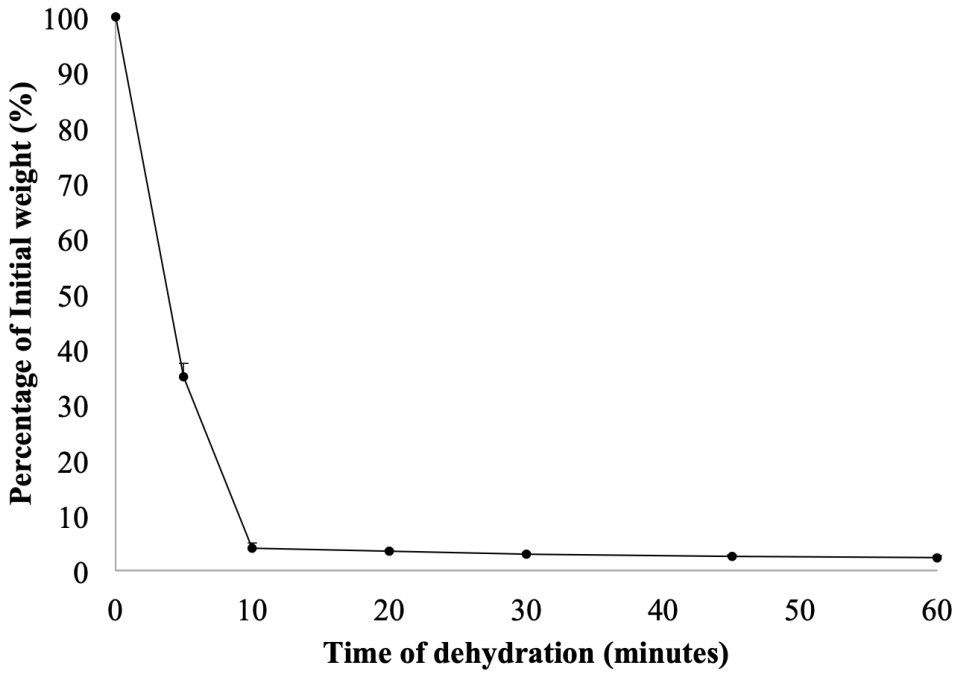


Figure 5.5: Percentage of initial weight after dehydration with sodium polyacrylate (10% w/w) for varying times. n=4, mean + SEM. Standard error within marker at 20, 30, 45 and 60 minutes.

5.3.2 Mechanical testing of dehydrated collagen gels with and without cells

A method of mechanically testing hydrogels in tension was developed as described in section 5.2.3. Collagen hydrogels were prepared with and without cells in specially designed and fabricated casting plates. The initial method, in which the 8 ml collagen gel was cast in one step, proved difficult to repeat. Before setting, sections of nylon mesh were added to the gels (Figure 2.4) to assist the gripping of the test sample in the test rig, however, several of the gels became detached from their embedded mesh during dehydration or transfer to the testing rig. An alternative method using a stainless steel mesh was tried, however, this also created problems with repeatability as the warp and weft of the stainless steel mesh caused large

differences in surface height, due to the thickness of the mesh, which itself initiated tearing of the gel. The method was improved by using a much thinner nylon cell strainer mesh during dehydration, which reduced the tearing. A sandwich of the mesh between two layers of collagen gel enclosing the gripping mesh (Figure 5.4) reduced detachment of the mesh prior to testing.

For the initial method of casting in a single step, 4 hydrogels were prepared in each condition. Gels contained 3 x 10^4 cells/cm^2 or were prepared cell-free, then were left hydrated or dehydrated with 10% (w/w) SP for 5 minutes. At least half of each group did not survive the testing process, reflected in the n value presented (Figure 5.6). Dehydration for 5 minutes gave a 62% and 79% weight reduction in cell-free gels and gels with embedded cells respectively (Figure 5.6 (a)). When compared by a Student's t-test, there was no significant difference between dehydrated gels with or without cells ($p=0.33$) however there was a significant difference between the dehydrated gel with embedded cells and its hydrated counter-part ($p<0.05$). The sample number was too small to compare the hydrated and dehydrated gels without cells. Mechanical testing showed (Figure 5.6 (b)) an increased Young's modulus in dehydrated gels with and without cells. Without cells, the stiffness increased from 1.31 kPa to 6.34 + 0.29 kPa when dehydrated. With cells, stiffness increased from 1.42 + 0.018 kPa to 4.35 + 0.04 kPa when dehydrated. No significant difference ($p=0.09$) was found when the Young's modulus of dehydrated gels with and without cells was compared by t-test.

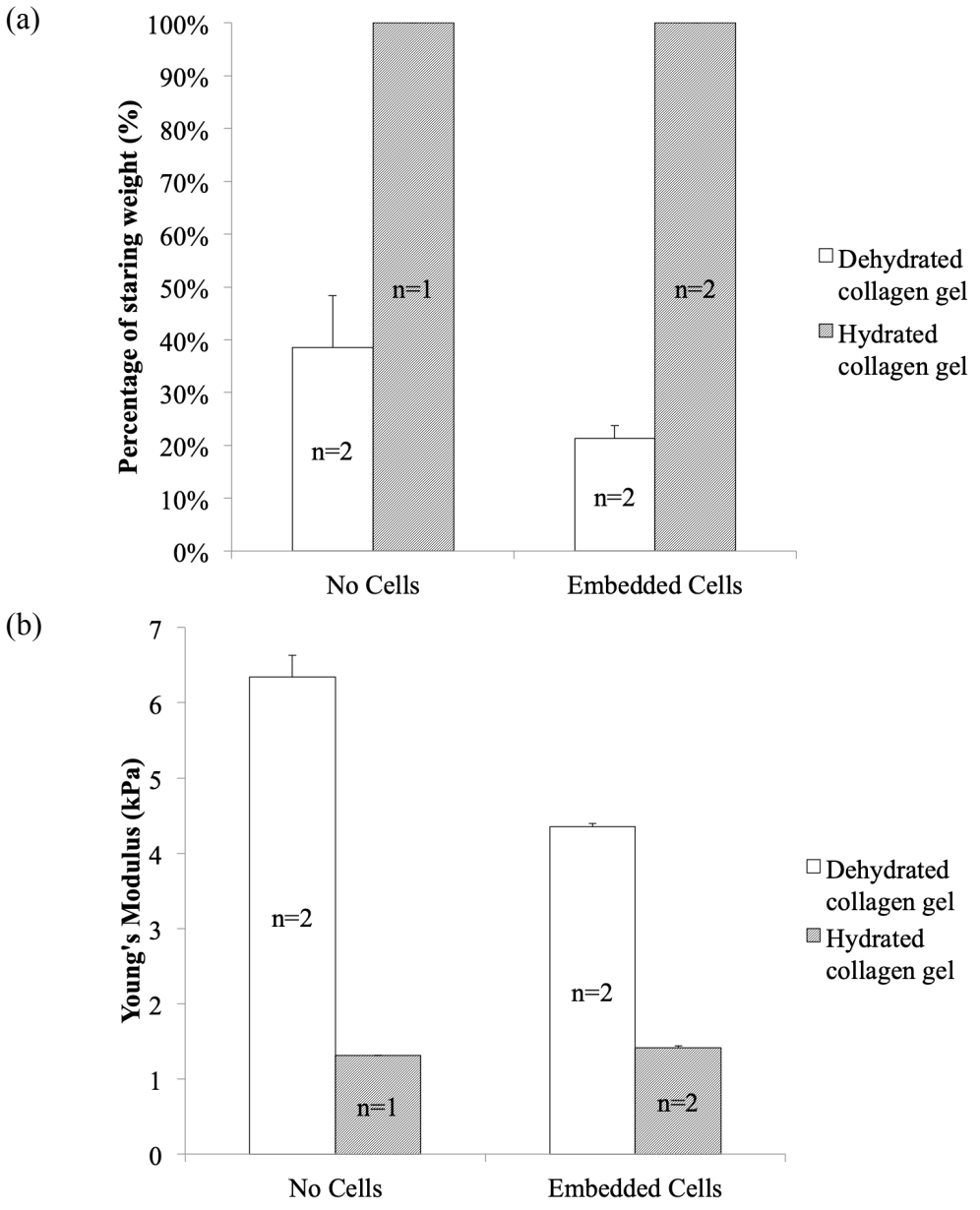


Figure 5.6 (a): Percentage of initial weight remaining after dehydration of collagen gels with or without cells with 10% (w/w) SP for 5 minutes. (b) Young's modulus, measured in tension. Mean + range.

The method was developed to include the mesh, to assist with gripping the sample, within a sandwich between an 8 ml and 2 ml collagen gel. This reduced the separation of gels from mesh prior to testing but increased the sample thickness. Collagen gels (0.3%) were prepared without the addition of cells then left hydrated,

or compressed with 10 or 20% (w/w) sodium polyacrylate. Four hydrogels were prepared in each condition, 2 of each of the dehydrated gels survived testing while all 4 hydrated gels survived. The weight and thickness of each gel after compression is displayed in Figure 5.7 (a). The thickness of the hydrogel closely followed the weight reduction. Gel weight decreased by 65% with 10% SP and 85% with 20% SP. The thickness of the gel started at 5.4 mm decreasing to 2.3 mm or 0.76 mm with 10 or 20% SP, equivalent to 60% or 86% reduction in thickness respectively. Young's modulus (Figure 5.7 (b)) increased with increasing dehydration, increasing exponentially on dehydration with 20% (w/w) SP. When compared by one-way ANOVA, there was a significant difference between the mean Young's modulus of hydrated, dehydrated (10% SP) and dehydrated (20% SP) ($p<0.001$) samples.

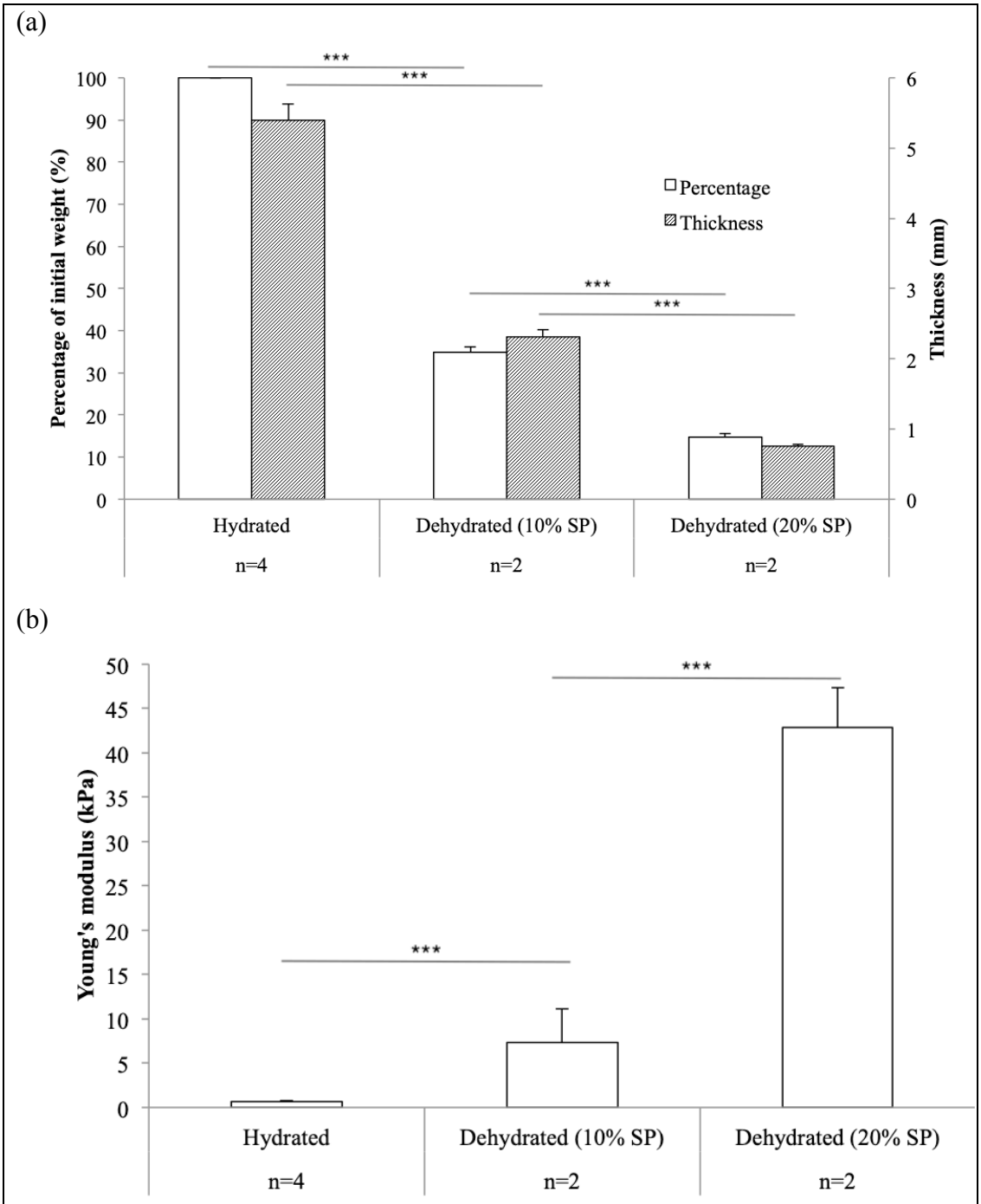


Figure 5.7: Percentage of initial weight and thickness of collagen hydrogel after dehydration with 10 or 20% (w/w) sodium polyacrylate. 6 hydrogels prepared for each condition without cells; n=4, 2 and 2 survived dehydration and mechanical testing. n=4: mean + SEM; n=2: mean + range. Results compared by one-way ANOVA, followed by Tukey's post-hoc analysis. * indicates *p<0.001.***

5.3.3 Viability of hydrated and dehydrated collagen hydrogels as long and short-term scaffolds for human dermal fibroblasts

To measure cell viability, an MTT assay was carried out on a sectioned area of the gel. As MTT is a colorimetric viability assay for cell metabolism, the absorbance was compared to a standard curve of number of human dermal fibroblasts against absorbance of MTT formazan formed. The MTT assay showed viable cells in both hydrated and dehydrated gels after 1 and 4 weeks (Figure 5.8). As can be seen, there was a significant increase in cell number in both gel preparations from initial seeding to 1 week and from 1 week to 4 weeks suggesting cells were dividing. When statistically compared by t-test, there was a significant increase in cell numbers between hydrated and dehydrated gels after 1 week *($p<0.05$)*. However, after incubation for four weeks, cells in dehydrated gels had recovered and there was no significant difference between the hydrated and dehydrated gels *($p=0.35$)*. Statistics are displayed in Appendix: Figure i.ii.

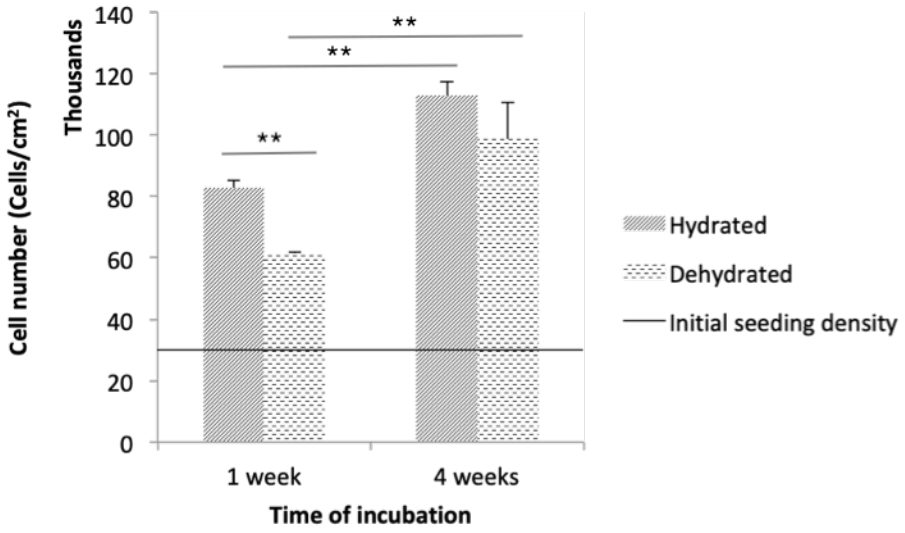


Figure 5.8: Collagen hydrogels (0.3%) prepared with HDFs then dehydrated with sodium polyacrylate (10% w/w). After 1 or 4 weeks in culture, an MTT assay was used to measure number of cells. The solid line signifies initial seeding density (30,000 cells/cm^2). n=4, mean + SEM. A Student's T-test was used to compare results. ** indicates *p<0.01.* There was no significant difference in the cell number contained in hydrated and dehydrated gels at 4 weeks (*p=0.35*).

To assess the viability of hydrated and dehydrated collagen hydrogels for the long and short-term culture of HDFs, collagen gels were prepared in a number of ways (Table 5.1). Hydrated and dehydrated collagen gels prepared without any additive, with ascorbic acid, with hyaluronic acid and with both ascorbic and hyaluronic acid were maintained for 1 or 4 weeks in culture. HDFs were added to the hydrogels prior to setting, at a density of 3 x 10^4 cells/cm^2. A set of cell-free controls was also prepared. Four samples were prepared by each method. Collagen gel weight, cell viability, collagen degradation, new collagen synthesis and cell morphology were all studied after 1 or 4 weeks.

Table 5.1: Preparation conditions of collagen hydrogels prepared for long and short-term support of HDFs. Collagen gels were prepared with or without human dermal fibroblasts and additives then left hydrated or dehydrated with SP. Each group maintained for 1 or 4 weeks, n=4.

Dehydrated	Collagen	Collagen + AA	Collagen + HA	Collagen + AA + HA	+ HDFs
	Collagen	Collagen + AA	Collagen + HA	Collagen + AA + HA	- HDFs
Hydrated	Collagen	Collagen + AA	Collagen + HA	Collagen + AA + HA	+ HDFs
	Collagen	Collagen + AA	Collagen + HA	Collagen +AA +HA	- HDFs

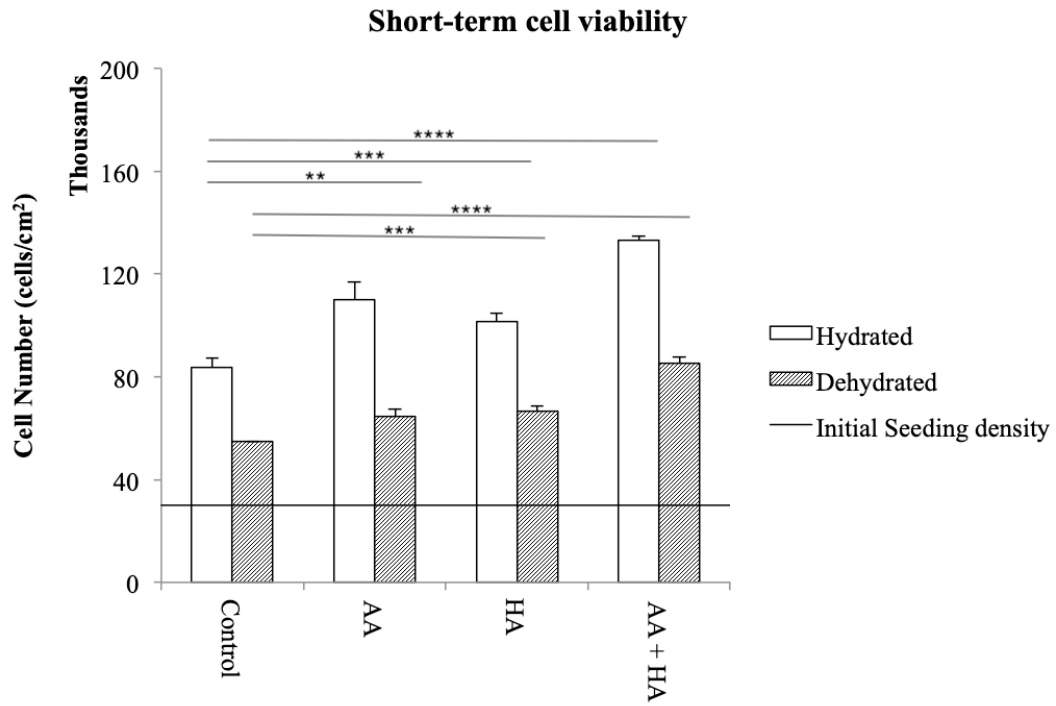
5.3.3.1 Cell viability in dehydrated collagen gels

The number of metabolising HDF cells was measured after 1 or 4 weeks in hydrated and dehydrated collagen scaffolds (Figure 5.9). Collagen scaffolds were initially seeded with $3x10^4$ HDFs/cm^2. After 1 week (Figure 5.9 (a)), cell number had increased significantly in all gels compared to the initial seeding level. Cell numbers in both hydrated and dehydrated hydrogels followed a very similar pattern with gels supplemented with AA displaying increased cell numbers compared to control gels and gels supplemented with HA while gels supplemented with both AA and HA

showed the greatest cell viability. Dehydrated gels contained fewer cells than hydrated gels overall. In the longer term (Figure 5.9 (b)), cell numbers had recovered in control gels when comparing hydrated and dehydrated gels. Gels supplemented with HA alone had a detrimental effect on cell number, while supplementing the hydrogels with AA seemed to improve cell viability. After 4 weeks, cell number remained greatest in gels supplemented with both AA and HA.

A statistical analysis was carried out in which a single variable: cell number, was compared with several variable factors by the general linear model with a confidence level of 95%. Three factors were considered: time point, hydration state and type of additive; as well as their interaction terms. Each individual factor had a significant effect on cell number ($p<0.001$) as did the interaction between time and additive. However the interactions between time and hydration; between type of additive and hydration; and between the three factors together was not linked to a significant difference in cell number (Appendix: Table i.iii). The results were further compared by one-way ANOVA followed by post-hoc analysis with a Dunnett's Test to compare each preparation with the relevant control. The results are presented in Figure 5.9. Hypothesis testing by one-way ANOVA was conducted, and the results are presented in Appendix section i.iv.i.i.

(a)


Short-term cell viability
Thousands
Cell Number (cells/cm²)
200
160
120
80
40
0
****
***
**
****
***
Control
AA
HA
AA + HA
Hydrated
Dehydrated
Initial Seeding density

(b)

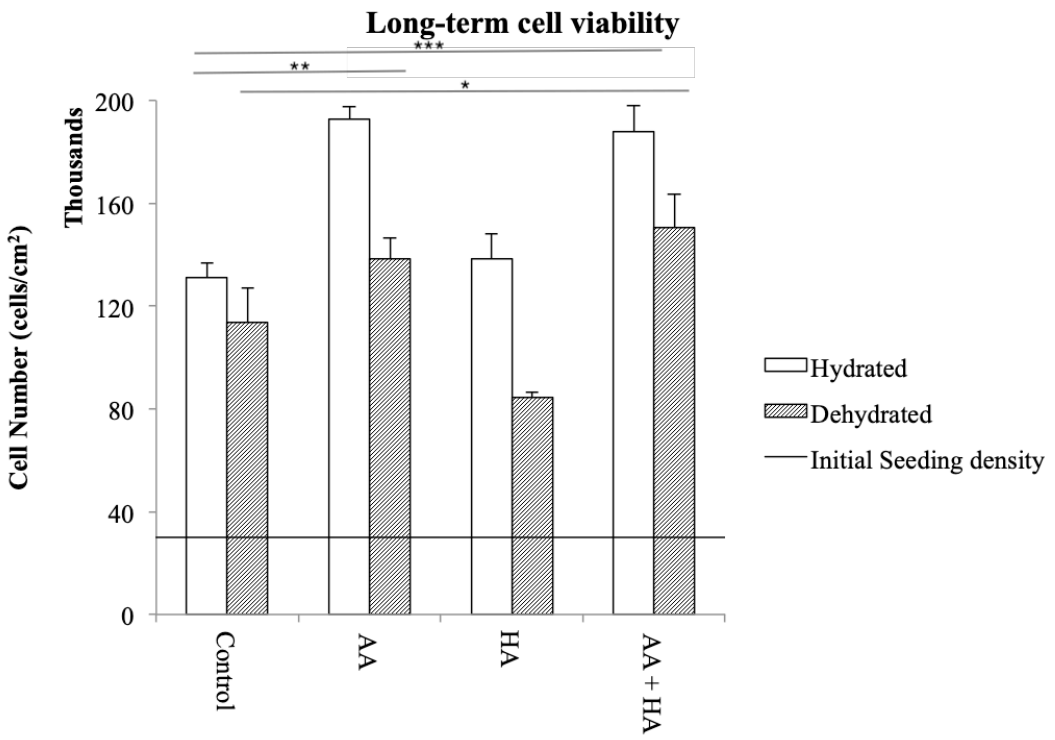


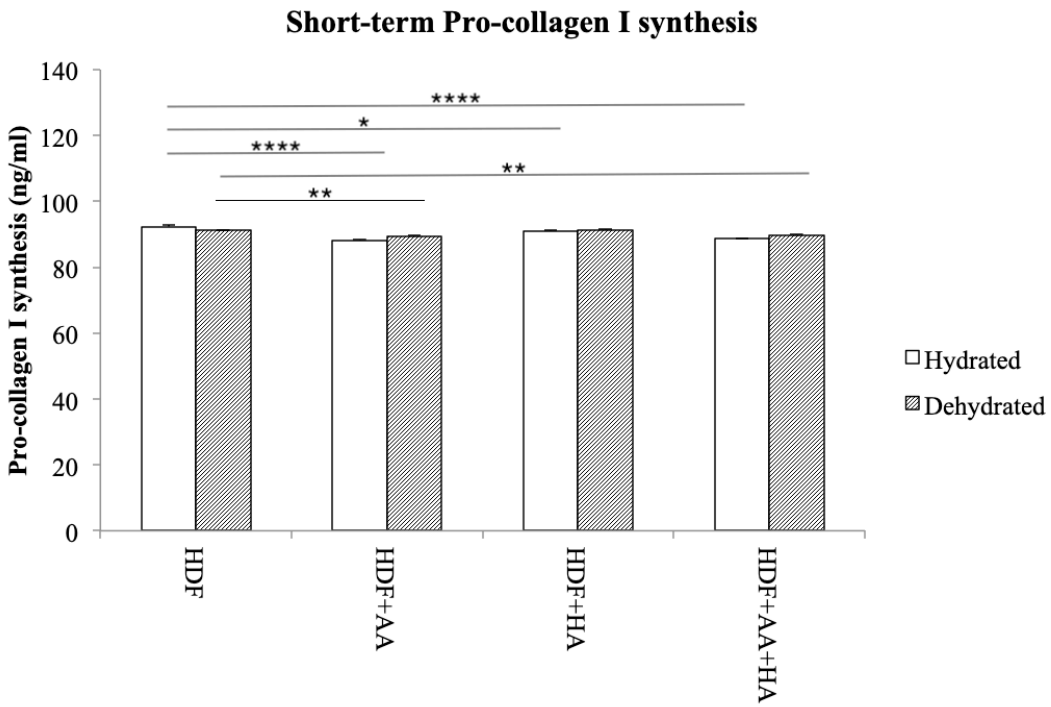
Figure 5.9: Cell viability in hydrated and dehydrated collagen hydrogels measured by MTT assay after (a) 1 week or (b) 4 weeks in culture. Gels were prepared with Human Dermal Fibroblasts then supplemented with: ascorbic acid (AA); hyaluronic acid (HA) or both. n=4, mean + SEM. Results compared by one-way ANOVA followed by Dunnett's post-hoc analysis, * indicates *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.*

5.3.3.2 Generation of new collagen by embedded fibroblasts

Pro-collagen, measured by ELISA, was used as a proxy to quantify de novo collagen synthesis in the samples (Figure 5.10). Each media change was collected and frozen until the end of the experiment. The frozen media were mixed, defrosted and centrifuged; then a sample tested to measure the concentration of pro-collagen present. After 1 week (Figure 5.10 (a)), all gels containing HDFs were found to be producing pro-collagen at a concentration of approximately 90 ng/ml of spent media.

None of the additives made any significant difference to the production of pro-collagen. By 4 weeks in culture (Figure 5.10 (b)), concentration of pro-collagen in control gels was significantly higher in the dehydrated gels (115.9 ± 6.3 ng/ml) compared to hydrated gels (68.6 ± 2.0 ng/ml). In hydrated gels, synthesis was greater in those gels supplemented with ascorbic acid, and hyaluronic acid. Ascorbic acid seemed to have the greatest impact, as supplementing with this alone increased synthesis to 100.2 ± 11.5 ng/ml, while hyaluronic acid alone increased synthesis to 85.0 ± 12.2 ng/ml. When hydrated gels were supplemented with both hyaluronic acid and ascorbic acid, collagen synthesis increased to 120.4 ± 1.3 ng/ml. The results for dehydrated collagen gels differed from those for hydrated gels as the un-supplemented control gels produced more de novo collagen than supplemented gels. Un-supplemented dehydrated collagen gels produced 115.9 ± 6.3 ng/ml of de novo collagen. Where ascorbic acid promoted synthesis in hydrated gels, it inhibited synthesis in dehydrated gels, gels supplemented with ascorbic acid producing 90.8 ± 6.8 ng/ml when added alone and 87.9 ± 2.2 ng/ml when added along with hyaluronic acid. Compared to ascorbic acid, hyaluronic acid inhibited synthesis less, giving a concentration of 94.9 ± 4.1 ng/ml. No *de novo* collagen synthesis was measured in gels prepared without fibroblasts. The results were compared by one-way ANOVA followed by post-hoc analysis with a Dunnett'sTest to compare each preparation with the relevant control. The results are presented in Figure 5.10. Further statistical analysis is presented in the Appendix: section i.iv.ii.

(a)


Short-term Pro-collagen I synthesis
Pro-collagen I synthesis (ng/ml)
140
120
100
80
60
40
20
0
****
*
****
**
**
HDF
HDF+AA
HDF+HA
HDF+AA+HA
Hydrated
Dehydrated

(b)

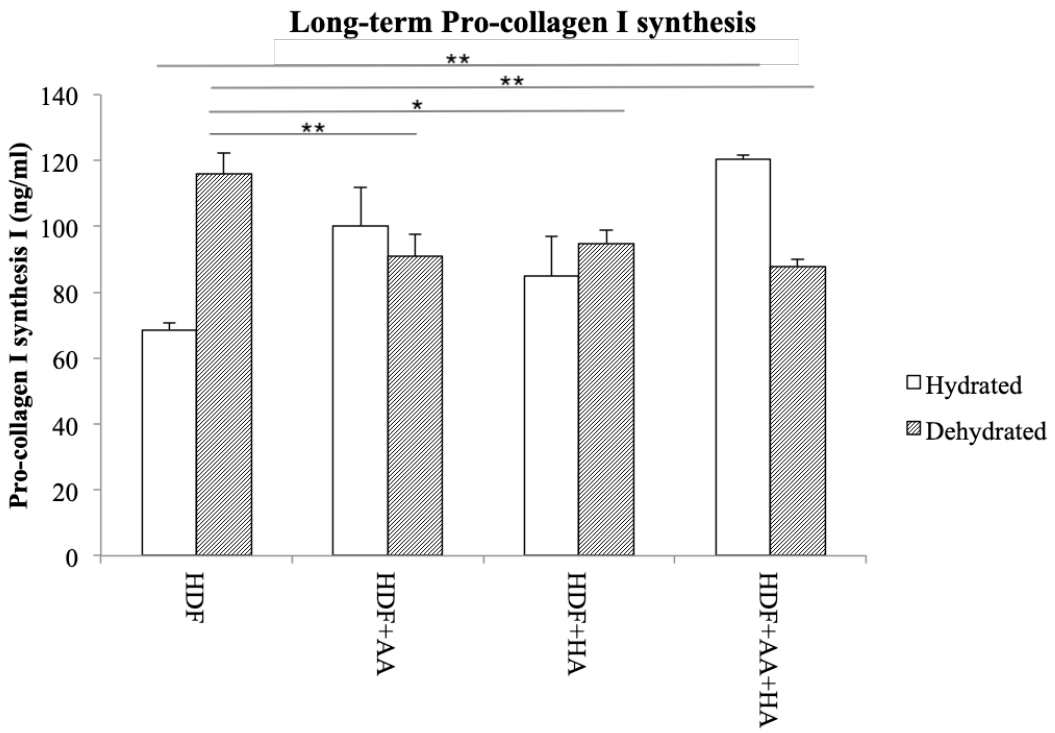


Figure 5.10: Concentration of newly synthesised collagen present in media changes after (a) 1 week or (b) 4 weeks, measured by pro-collagen I ELISA. Gels were prepared with Human Dermal Fibroblasts (HDF) then supplemented with: ascorbic acid (AA); hyaluronic acid (HA) or both. n=4, mean + SEM. Results compared by one-way ANOVA followed by Dunnett's post-hoc analysis, * indicates *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.*

Interestingly, collagen synthesis didn't follow cell number. Concentration of collagen normalised to cell number (Figure 5.11) shows the greater cell numbers at the four week point did not necessarily equate to increased collagen synthesis, although this could suggest that the rate of production of pro collagen, like the degradation of the lattice, slowed down as the experiment progressed. HDFs in dehydrated collagen gels all produced more pro-collagen per HDF than their hydrated counterparts.

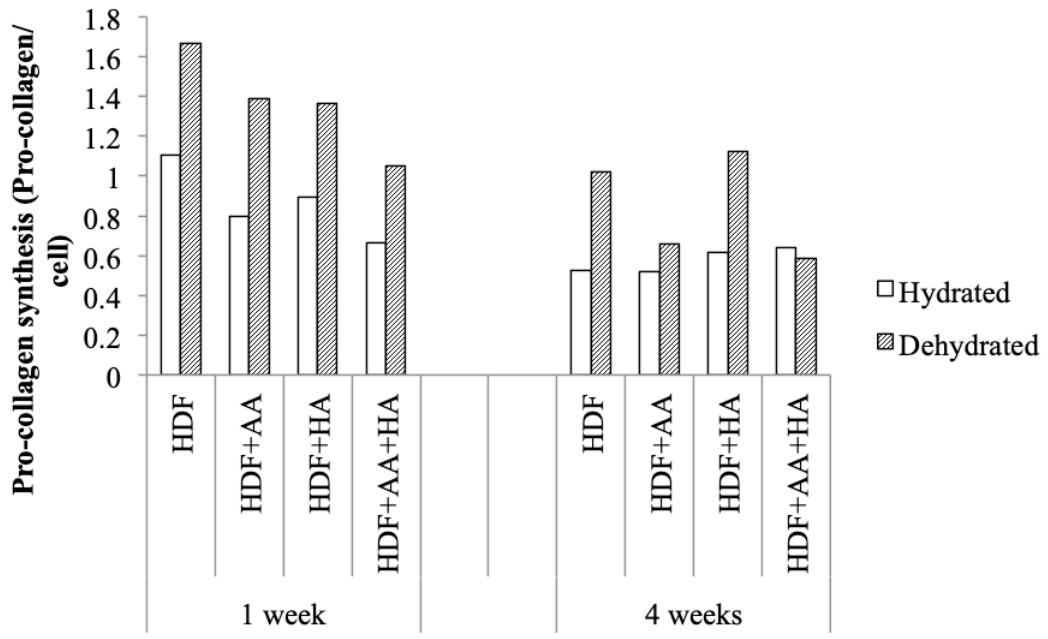


Figure 5.11: Concentration of pro-collagen synthesised (ng/ml of spent media) per HDF, after 1 or 4 weeks in culture. n=4. Collagen gels were supplemented with ascorbic acid (AA), hyaluronic acid (HA) or both.

5.3.3.3 Cell morphology

Cells in each gel preparation were examined by microscopy to visualise their morphology. Phalloidin-FITC (Green) was used to stain HDFs for actin; and nuclei counterstained with DAPI (Blue). After 1 week, samples were visualised with a Zeiss upright microscope fitted with an Apotome which optically sectioned the samples (Figure 5.12 (a,c,e,g) & Figure 5.13 (a,c,e,g)). A Leica confocal microscope was used to image the samples after 4 weeks (Figure 5.12 (b,d,f,h) & Figure 5.13 (b,d,f,h)).

At both time points, cells were present throughout both hydrated and dehydrated gels. In hydrated collagen gels (Figure 5.12), the HDFs had a rounded morphology, especially in the middle layers of the sample; whereas in dehydrated samples, they

had a much more fibroblastic morphology with greater actin spreading (Figure 5.13). There was significant variation in cell number through the depth of the hydrated hydrogel, with some depths having very sparse cell growth (Figure 5.12 (a,e)). The cells in dehydrated gels were much more densely packed and therefore showed some cell-to-cell contact (Figure 5.13 (c)). After 4 weeks, all dehydrated gels showed a confluent layer of fibroblasts (Figure 5.13 (b,d,f,h)), while the hydrated gels remained sparse (Figure 5.12 (b,d,f,h)). The number of cells, measured by MTT seemed to correspond with the density of cells in the microscopy images.

Hydrogel thickness was measured at 4 weeks using the confocal microscope. The lower surface of the sample was brought into focus and z-height noted, then the upper surface focused and the difference in z-height calculated. The thickness of hydrated samples was approximately 600 μm compared to ~60 μm in dehydrated samples. All dehydrated samples after 1 and 4 weeks showed the reverse topography of the nylon mesh used during dehydration (Figure 5.13 (b,d,f,h)).

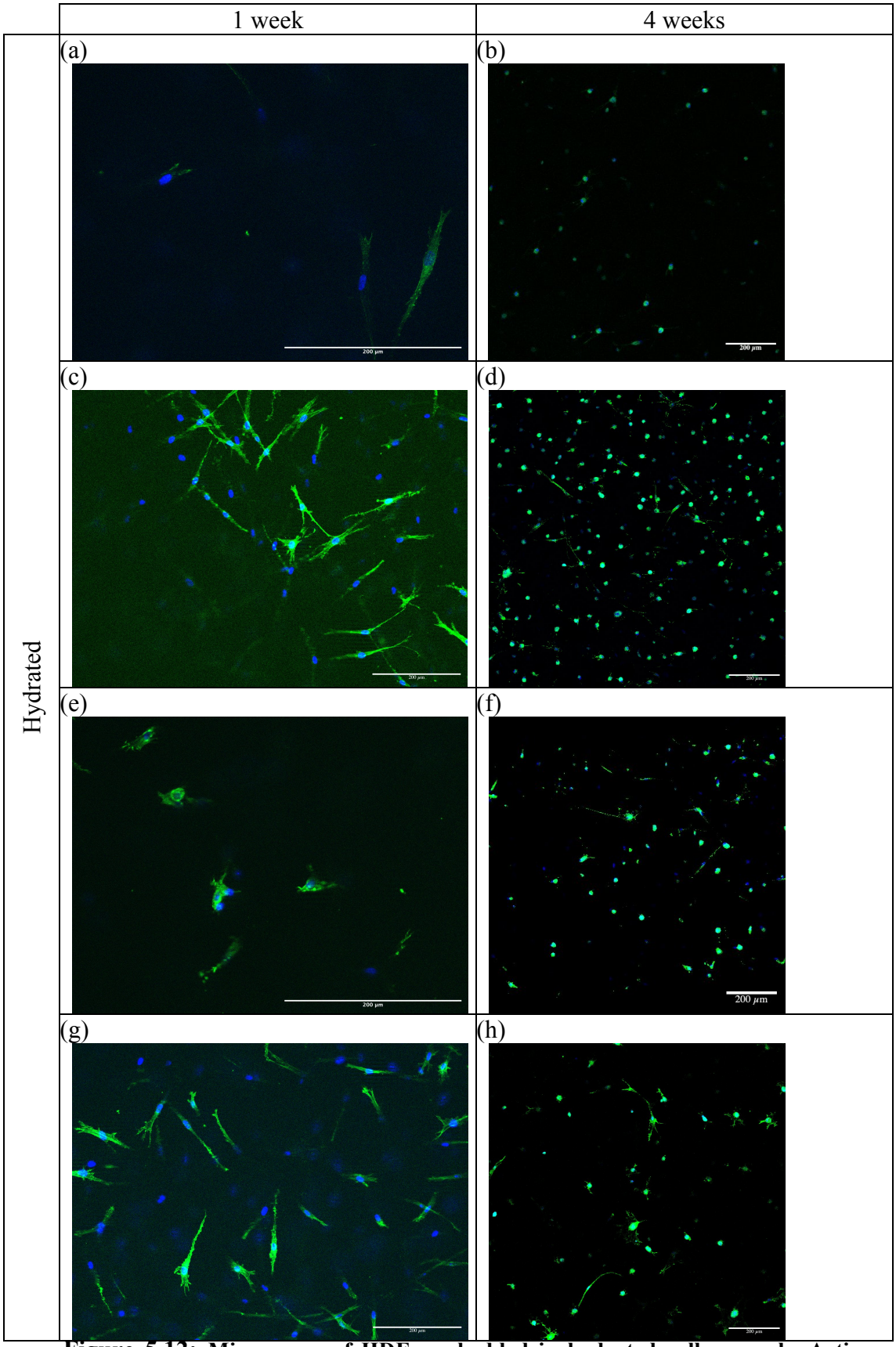


Figure 5.12: Microscopy of HDFs embedded in hydrated collagen gels. Actin stained with phalloidin (green) and nuclei counterstained with DAPI (Blue). (a,c,e,g) imaged after 1 week and (b,d,f,h) imaged after 4 weeks in culture. Gels were (a,b) unsupplemented; (c,d) supplemented with ascorbic acid; (e,f) supplemented with hyaluronic acid; and (g,h) supplemented with both ascorbic acid and hyaluronic acid.

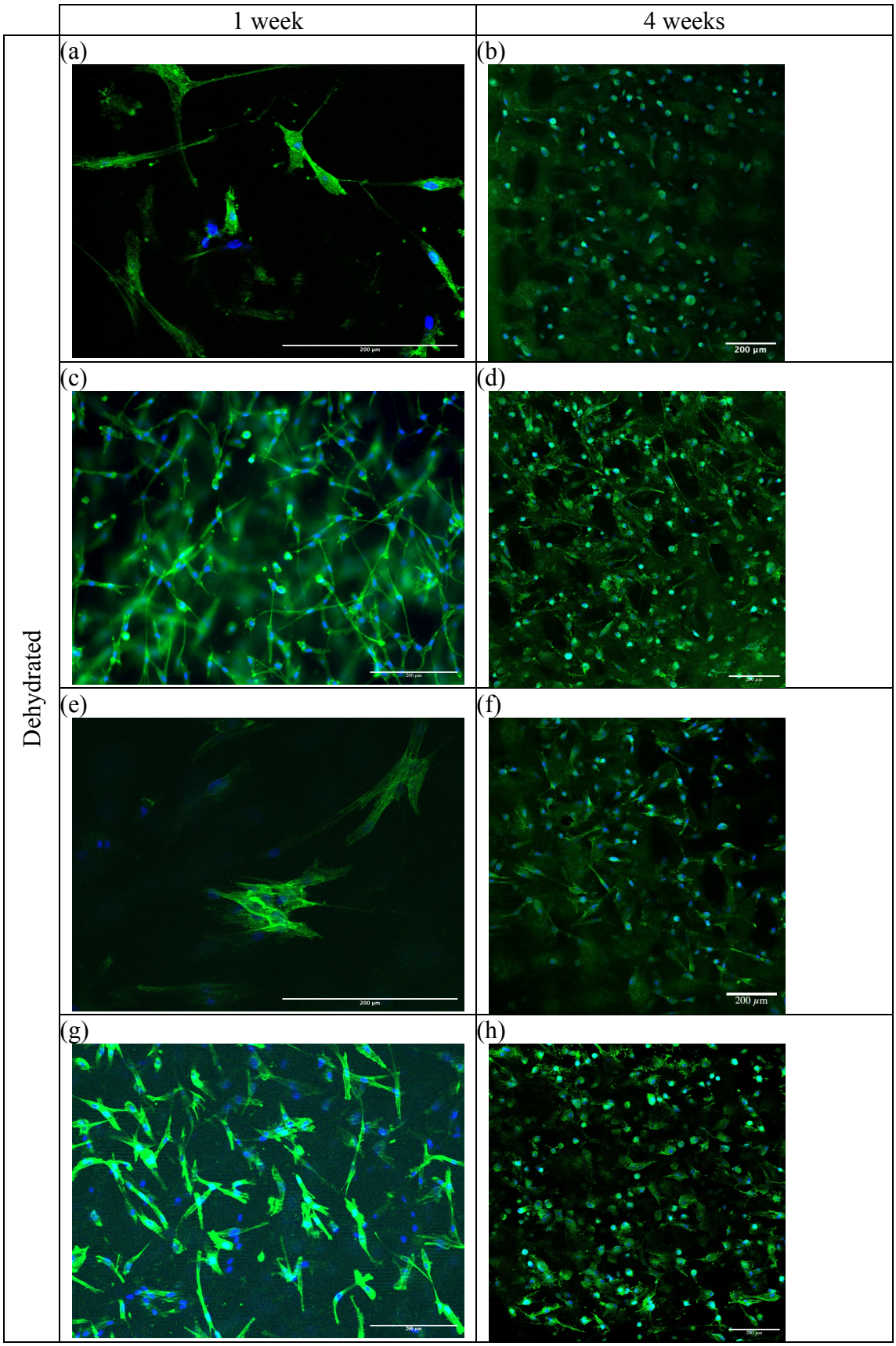


Figure 5.13: Microscopy of HDFs embedded in dehydrated collagen gels. Actin stained with phalloidin (green) and nuclei counterstained with DAPI (Blue). (a,c,e,g) imaged after 1 week and (b,d,f,h) imaged after 4 weeks in culture. Gels were (a,b) unsupplemented; (c,d) supplemented with ascorbic acid; (e,f) supplemented with hyaluronic acid; and (g,h) supplemented with both ascorbic acid and hyaluronic acid.

5.4 Discussion

The work in chapter 4 showed that supplementing collagen hydrogels with ACE inhibitors did not lead to observable bulk changes in mechanical properties, so other methods of manipulation were explored. Plastic compression was developed by Robert Brown's group in UCL (Brown et al., 2005) as a method to improve the mechanical properties of collagen hydrogels by forcing water out of the set gels, primarily through a combination of mechanical pressure and capillary action. It has been widely used and modified (Abou Neel et al., 2006; Mi et al., 2010; Alekseeva et al., 2011, 2012; Cheema and Brown, 2013). Lonza commercialised the Raft system, based on the method from the Brown group (Lonza, 2019). There are many laboratories in the UK, and abroad, which now use the Raft system to produce 3D cell laden collagen scaffolds. These scaffolds are useful for numerous research applications, such as corneal models for ocular research, skin models for dermal research and liver models for research on fibrosis, to name but a few. The system is useful in concentrating collagen while maintaining around 60% of the initial cell number post dehydration. However, this system is fairly expensive, costing around £105 to dehydrate a 24 well plate and is not obviously scalable to use in in-vivo, rather than laboratory based applications. This chapter focussed on investigating a new method of plastic compression to ascertain whether it could improve cell viability while reducing the associated cost considerably.

5.4.1 Dehydration of collagen gels

As previously stated, collagen is an excellent biomaterial for cellular applications. It is readily available, synonymous between species and is biologically active,

promoting cell growth and adhesion. The main drawback with collagen hydrogels is their mechanical instability - they are not able to support their own weight when removed from cell culture medium. Isolation of collagen can be achieved by many methods, the main ones being acid or pepsin solubilisation. Both of these methods limit the achievable concentration in the final collagen solution, limiting the maximum collagen concentration in a hydrogel to around 0.6%, a concentration which represents a mechanically weak gel. There are several known ways to improve the mechanical properties of hydrogels; all work either by crosslinking the collagen fibrils or by increasing the concentration of collagen in the gels (Roberts and Martens, 2016). Crosslinking collagen hydrogels often involves the use of toxic chemicals, which prevents the inclusion of cells prior to setting, thus precluding true three-dimensional culture required when recapitulating biological tissues (Table 1.2). Plastic compression was designed by Robert Brown's laboratory to overcome this. The initial method of plastic compression forced water out of hydrogels by removing the hydrogel from a casting chamber, placing it on absorbent paper and placing a glass plate on its upper surface with weights added to the upper surface of the plate (Brown et al., 2005). This successfully produced thin layers of collagen containing viable cells using gravity to force water in a single direction onto the absorbent paper below. To further concentrate the collagen, the compressed collagen sheet was rolled from the short axis and compressed again. This was successful in producing bars of multiple layers of collagen, however, was not conducive to maintaining any viable embedded cells (Bitar et al., 2007). Bitar and colleagues also found desiccation extremely affected cell viability, which could be the cause of the loss of cell viability in double plastic compression. Alternatively, the process of repeating the plastic

compression could be overly traumatic for embedded cells in dehydrated gels. The plastic compression method was further adapted to use a special machine to roll absorbent paper to produce a plunger that could be placed on the surface of hydrogels to draw the water from the upper surface of hydrogels. This method was commercialised by Lonza (Lonza, 2019). The drawbacks of this method are that it is expensive and its use is limited to a well of a 24 or 96 well tissue culture plate. Cell populations embedded in the Lonza raft system have 60% viability when measured by Live Dead assay immediately after compression. In this chapter, a new method to cheaply and reliably dehydrate collagen gels retaining cell viability was developed. This method would also be easily scaled up.

Sodium polyacrylate (SP) was chosen as a super-absorber to dehydrate collagen gels, as it is used widely in personal hygiene products and food, and approved by the FDA and CIR for use in food for human consumption or personal care products (Fiume, 2002). It is also readily available and cheap. Dehydration of hydrogels using readily available nylon and metal meshes, with and without filter paper, and with various amounts of the super-absorber was explored. Using 10% (w/w) SP with nylon mesh and a single layer of filter paper proved the most repeatable of the combinations tested and showed that dehydration proceeded rapidly and reliably with time (Figure 5.5). A 96% weight reduction was achieved in the first 10 minutes of dehydration, increasing to 98% by 60 minutes with no mechanical compression of the gel beyond the weight of the filter paper and the SP. Using increasing amounts of SP accelerated the process further (Figure 5.7). During the method development, the super-absorber was stained with Sirius Red to test for the presence of collagen, however, no collagen was found indicating the super-absorber did not absorb the collagen scaffold. In early

development of the method, a metal mesh was used to separate the gel from the super-absorber. Replacing this with nylon mesh, which had thinner fibres, reduced attachment of the mesh and gel and thus avoided damage to the gel. The nylon mesh was cut to the size of the gel from a large sheet, consequently scaling up or down could be easily accommodated by this method. A layer of filter paper was placed between the SP and nylon mesh to prevent any of the SP attaching to the gel and enhancing localised dehydration.

It was imperative to also reduce handling of the hydrated hydrogels during the dehydration process. Their fragility meant the gels were easily damaged during removal from a casting plate. This meant that gels had to be produced in excess, thus increasing the cost and time of the procedure. During development of the method, casting, dehydrating and maintaining the gels in a single sterile tissue culture plate, reduced handling of the hydrated gels dramatically. Previously, with repeated handling and movement from each stage, there was an increased risk of compromising the sterility of the gel, thus affecting its usefulness as a technique for cell culture. Casting, dehydrating and maintain the gels in a single tissue culture plate came with the added benefit that the tension in the gel was maintained by remaining attached to the sides of the tissue culture plate, which was impossible when moving it during previous iterations of the method. The reduced handling also prevented damage to the gel, improving its mechanical integrity and stability. Further improvement could be made to the technique by creating devices which allow easy application and removal of the mesh and super-absorber in one-step, however this

begins to add the need for further technical equipment and reduces the flexibility of offered by the current method.

5.4.2 Mechanical testing of dehydrated collagen gels

Ultimately, the aim of this work was to produce a skin-like scaffold to be used as a dermal tissue graft. It is crucial that a skin scaffold has tensile mechanical strength as skin is in constant tension in the body. As all mechanical testing previously completed (Chapter 3 & 4) had been of gels in compression, a method to mechanically test in tension was developed. The initial method, featuring a single layer of collagen embedded with cells and with a thin nylon mesh, to assist with gripping of the gel by the testing rig, showed mechanical stiffness increased dramatically after dehydration (Figure 5.6). Dehydrated gels prepared without cells had a Young's modulus of 6.3 kPa compared to those prepared with fibroblasts which had a Young's modulus of 4.4 kPa. These were both significantly stiffer than hydrated gels (with and without fibroblasts), which both had a stiffness of 1.3 kPa and 1.4 kPa, respectively. The increased stiffness was approaching that of a useful strength for tissue analogues; the elastic modulus of skin having been measured by Zahouani and colleagues as 8.2 kPa (Zahouani et al., 2009). The stiffness of a cell scaffold has been shown to be an important factor in scaffold design, particularly in controlling the morphology of cells and guiding stem cell development on such scaffolds (Park et al., 2011; Chen and Jacobs, 2013). Mesenchymal stem cells took on a neurogenic lineage on soft matrices while those seeded on hard matrices took on a myogenic lineage (Ho et al., 2017). Human dermal fibroblast proliferation was found to be dependent on matrix stiffness (Hadjipanayi et al., 2009), and their

morphology was radically different on soft or stiff matrices. On soft matrices, HDFs remained rounded while stiff matrices gave rise to a stretched morphology with an organised cytoskeleton which proliferated normally (Discher, 2005; Ghosh et al., 2007). It is therefore important to be able to choose a scaffold material that matches, as far as possible, the in vivo situation the scaffold is being used to mimic.

Many of the collagen gels in the first round of testing became detached from the mesh embedded to assist in gripping the hydrogels. This was observed to occur because the mesh floated to the surface of the hydrated gel, and so was only loosely attached to the gel. Therefore the method of mechanical testing was further adapted to allow the collagen to set before embedding the mesh and adding a second collagen layer. Two levels of dehydration were tested, using 10 or 20% (w/w) SP for 5 minutes. This improved the rate of survival of gels throughout the testing process and showed that increasing dehydration also increased the mechanical stiffness, ultimately reaching an elastic modulus of 43.2 ± 5.4 kPa with 85% dehydration. This massive increase in stiffness led to much easier handling as well as to increased cell viability and improved cell morphology on the scaffolds, as discussed in the following sections.

5.4.3 Cell viability in dehydrated collagen gels

Cell viability was measured by MTT assay, an assay of cell metabolism. Cell number was inferred from increased cell metabolism. Cells were initially seeded within the gels at a density of 30,000 cells/cm^2 of the gels' surface area. A measure of cell density as a function of area automatically accounted for the reduction in thickness

of the gel as it dehydrated to better compare the total cell numbers in hydrated and dehydrated gels. The dehydration process dramatically decreased the height, and thus volume of the hydrogels, therefore cells were condensed into much more compacted cell layers. Cell number in hydrated and dehydrated control gels were initially compared to assess the effect of the dehydration process on the cells. After 1 week, hydrated gels contained approximately 80,000 cells/cm^2 while dehydrated gels contained 60,000 cells/cm^2. There was a significant difference between the cell numbers, however in both preparations, there was an even more significant increase in the number of cells relative to the initial seeding. This suggests that the ability of cells to proliferate was not affected by the dehydration process even if some cells were lost during the process. By 4 weeks, there was no significant difference in number of cells between hydrated and dehydrated control gels, with both gels containing around 140,000 cells/cm^2. The number of cells in dehydrated gels after 1 week was significantly less than in hydrated gels, however converged with the hydrated gels after 4 weeks. This suggests that the rate of proliferation of cells likely increased in dehydrated gels compared to those in the hydrated gels as the time in culture progressed. Alternatively, cell proliferation slowed more in hydrated gels than in their dehydrated counterparts. As mentioned in 5.4.2, it has previously been shown that fibroblasts are more inclined to proliferate on stiffer gels. It is likely that completing the production, polymerisation, and dehydration of the gel then maintaining the cell culture in a single tissue culture plate as described above maintained the hydrogel in tension throughout. Kono and colleagues showed that fibroblasts in free-floating gels were more likely to undergo apoptosis than those kept in tension (Kono et al., 1990; Grinnell, 1994; Hadjipanayi et al., 2009). When

hydrogels are maintained in tension, fibroblasts seeded within the matrix are akin to those in granulation tissue during wound healing so rapidly divide and produce new extracellular matrix (Clark, 1993). Morphology showed results consistent with the literature; with sparse cell growth of rounded cells in soft hydrated gels and confluent layers of fibroblastic cells in stiffer scaffolds. Microscopy also showed the topography of the nylon mesh used during the dehydration process remained after 4 weeks. This could be exploited in the future as topography has been widely shown to improve the viability of cultured cells.

5.4.4 Supplementing dehydrated collagen gels with glycosaminoglycans (hyaluronic acid) and ascorbic acid

Ascorbic acid (AA) was chosen as an additive to study its effect on improving cell viability in the matrix as it has previously been shown to have a positive effect on cell proliferation (Boyce et al., 2002; Pullar et al., 2017). As AA is also a co-factor in collagen synthesis, it was thought that addition of AA might also improve new collagen generation. The ascorbic acid was added to the gels in the media changes every second day to maintain a consistent concentration. After one week, gels supplemented with ascorbic acid contained greater cell numbers in both hydrated and dehydrated gels. The effect was more pronounced in hydrated gels than in dehydrated gels. After four weeks, gels supplemented with AA still contained more cells than both hydrated and dehydrated control gels. There were still significantly more cells in hydrated gels than in dehydrated gels both supplemented with ascorbic acid. This could have been due to cells in the larger volume of the hydrated gels

having, in reality, much more space to continue to proliferate whereas those in dehydrated gels were approaching 100% confluency (as shown by microscopy).

Hyaluronic acid (HA) was chosen as one of the most prevalent glycosaminoglycans present in human skin. As a molecule most commonly known for maintaining hydration in the skin, it was thought to be useful in dehydrated gels for maintaining hydration levels, conferring viscoelasticity and promoting cell proliferation. In hydrated gels, HA had a slightly beneficial effect on cell viability after both 1 and 4 weeks. In dehydrated gels, the HA had a minor beneficial effect after 1 week and a detrimental effect on cell number after 4 weeks, with dehydrated gels prepared with HA containing approximately 30% less cells than their equivalent control gels. Interestingly, in all gel preparations, when hyaluronic acid and ascorbic acid were added to gels together, cell viability improved more than when either supplement was added alone, except in hydrated 4 week gels where the addition of ascorbic acid alone gave the largest rise in cell number.

5.4.5 Degradation of dehydrated collagen gels by embedded fibroblasts

These experiments could have been extended to include a method of measuring degradation of the collagen scaffolds by embedded human dermal fibroblasts. Methods such as radio-labelling collagen or an antibody assay as described by Oostendorp and colleagues were explored, however these methods were not feasible within a reasonable timeframe (Oostendorp et al., 2016). Quantifying biomarkers of collagen and HA degradation, such as collagen metabolite C1M (Leeming et al., 2012; Bay-Jensen et al., 2019) or low molecular weight hyaluronan (Cowman et al.,

2015), by ELISA or metabolomics could also prove useful in measuring turnover of the ECM in scaffolds. It is imperative that further work to measure degradation of plastically compressed collagen hydrogels conclusively is completed.

5.4.6 Generation of new collagen by embedded fibroblasts

New collagen synthesis was measured by an ELISA assay. As rat-tail collagen was used in the gel preparations, then embedded with human dermal fibroblasts, an antibody could be employed to measure newly synthesised human collagen. Control gels showed no significant difference in collagen synthesis between hydrated and dehydrated collagen gels after one week. After 4 weeks, however, collagen synthesis in dehydrated gels containing HDFs was almost double that of hydrated collagen gels. After 1 week, there are significantly fewer cells in the dehydrated gel than in hydrated gels whereas after 4 weeks, there is a negligible difference in cell number between the hydrated and dehydrated preparations. This suggests that the dehydration of collagen gels induced enhanced synthesis of de novo collagen by HDFs, as fewer cells in the dehydrated gels produced greater concentrations of pro-collagen than the hydrated collagen gels, which contained more cells. In the short term, there was no difference in collagen synthesis between control and supplemented hydrated and dehydrated gels. The results from the 4 week measurements suggest a different story, with gels supplemented with ascorbic acid, hyaluronic acid and both supplements together, enhancing pro-collagen synthesis in all hydrated gels. Ascorbic acid seems to have the greatest effect on collagen synthesis, as when added alone and with hyaluronic acid synthesis increases from ~70 ng/ml to 100 ng/ml and to 120 ng/ml, respectively. Supplemented dehydrated

collagen gels show reduced concentrations of pro-collagen compared to control dehydrated gels. When pro-collagen concentration was compared to cell number, HDFs in dehydrated gels were producing greater concentrations of pro-collagen than those in hydrated gels at both 1 and 4 weeks.

5.4.7 Conclusions from controlled dehydration study

This chapter demonstrated the viability of a novel method of dehydrating collagen hydrogels both quickly and reproducibly, so improving their mechanical properties. The described method is extremely promising for further study and potential practical application. Current methods of improving the mechanical properties of collagen hydrogels by forced dehydration, whilst effective in improving stiffness without compromising cell viability, are expensive, limited in application to small scale samples and require repeated handling of the gel, increasing the risk of both mechanical damage and compromising sterility. The method described here, using the super absorber sodium polyacrylate, proved highly effective for dehydrating collagen gels quickly and reproducibly whilst having minimal effect on the viability of embedded cells. As the preparation, polymerisation, dehydration and subsequent cell culture can all be completed in a single standard polystyrene tissue culture plate, the handling of the gel is greatly reduced. This helped retain the sterility of the gel whilst also maintaining tension within the gel, potentially improving cell proliferation (Grinnell et al., 1999; Hadjipanayi et al., 2009), a desirable feature for in vivo applications. Measurements showed that fibroblasts embedded in dehydrated gels produced greater concentrations of pro-collagen and proliferated faster than those in hydrated gels. This suggests the fibroblasts contained in the dehydrated collagen gels are more like those contained in granulation tissue than those contained

in the extremely soft hydrated scaffolds. This is very useful as a model for wound healing, or in future uses of this technology for dermal scaffolds, where embedded fibroblasts could support wound healing. There is significant scope for further research on the degradation of the original scaffold material using either radiolabelling or biomarkers of collagen metabolism to further understand the mechanism and rate of degradation of the scaffold material. If degradation could be controlled, either by addition of pharmaceuticals or manipulating collagen concentration in dehydrated gels, to correspond to *de novo* collagen synthesis and cell proliferation, these scaffolds could be an incredibly useful tool in the future of wound dressing technology.

Adding HA and AA together, further increased cell viability and pro-collagen synthesis, and improved cell morphology, all highly desirable properties for in vivo applications. The apparent synergistic effects of adding HA and AA to gels together suggest that there is scope for additional research to explore the interactions of adding other GAGs to dehydrated gels. Chondroitin sulphate, dermatan sulphate, heparin sulphate and keratan could all have beneficial biomimetic effects for skin scaffolds. This research also suggests that a positive avenue to explore would be to further refining the dehydration technique to create dehydrated hydrogels with similar concentrations of collagen to natural skin. Microscopy after 4 weeks in culture showed the hydrogels retained the topography of the nylon mesh, used during dehydration. This could be exploited by using a pattern mask during the dehydration process instead of a nylon mesh. Adjusting surface topography could also be useful in creating scaffolds with the ability to support a co-culture. Fibrous collagen

scaffolds with topographical cues have previously been shown to induce MMP production and enhance re-epithelialisation when culturing keratinocytes *in vitro* (Fu et al., 2014; Bernstam et al., 1990). Natural skin is a layered structure, with each layer contributing to give function as a whole. A bi-layered scaffold maintained in tension, consisting of a collagen dense fibroblast rich environment with surface topography to support a stratified epidermis of keratinocytes would be incredibly useful in the treatment of full thickness burns, diabetic ulcers or sites of injury.

Chapter 6

Summary and further work

6 Summary and Further Work

6.1 Key findings

Collagen is the most abundant protein in the ECM and is highly conserved between species. This makes it an excellent choice of biomaterial for a variety of biomedical and tissue engineering applications as it is highly biocompatible and weakly immunogenic. There are many methods of preparation which can produce biomaterials based on the collagen molecule such as electrospun nanofibres, lyophilised sponges, collagen films and scaffold hydrogels. The work in this thesis focussed solely on collagen hydrogels produced from type I collagen. Collagen hydrogels can be modified in many ways depending on the desired purpose, such as adding drugs for controlled release and addition of synthetic polymers to control degradation. The most significant disadvantage of collagen hydrogels is their weak mechanical strength; they are unable to support their own weight when removed from fluid.

The overarching theme of this work was an investigation of methods to increase the mechanical strength of collagen hydrogels and to maintain or enhance their biocompatibility for use as cell scaffolds. The work was primarily targeted towards dermal applications such as wound dressings and dermal equivalents, however improving the mechanical stability of hydrogels would be beneficial in a wide range of other tissue engineering applications. Three independent approaches were explored.

Firstly, a baseline comparison of collagen sourced from different species, and isolated by different methods, was undertaken to understand how the stock biomaterial differed in terms of fibril formation and compressive mechanical strength. The behaviour of cells embedded in the different hydrogels was then studied using a common wound healing model developed by Bell and colleagues, incorporating fibroblasts from the 3T3 murine cell line into free floating hydrogels and observing contraction profiles.

The second approach incorporated drugs from the ACE inhibitor family in the collagen hydrogels. ACE inhibitors have been shown previously to inhibit degradation of collagen by MMPs. Three ACE inhibitors were chosen, and their effects on cell viability, gel stiffness and fibroblast contraction were assessed to investigate the effect of incorporating the drugs on both the biocompatibility and the mechanical degradation of the treated hydrogels.

Finally, incorporating the super-absorber, sodium polyacrylate into the dehydration process developed a novel method of dehydrating hydrogels. This proved extremely effective in rapidly increasing mechanical strength of the gels without significantly adversely affecting the resident cell population. This technique was used to produce hydrogels containing viable human dermal fibroblasts. In further studies ascorbic acid and hyaluronic acid were included in the dehydrated hydrogels and were found to further enhance their biocompatibility. The resulting hydrogels were assessed for cell viability, newly synthesised collagen and the cell morphology imaged.

The detailed research findings from each approach are covered in this thesis as follows:

Chapter 3: Comparison of collagen types isolated from different sources

Chapter 4: Manipulating collagen properties with ACE inhibitors

Chapter 5: Controlled dehydration of collagen hydrogels

6.1.1 Comparison of collagen types isolated from different sources

The properties of gels produced from three collagen preparations (acid solubilised rat-tail tendon (ASRT); pepsin solubilised bovine dermis (PSB); and pepsin solubilised rat-tail tendon (PSRT)) were compared with a view to establishing working methods and whether results could be translated between samples prepared from different sources. The mechanical stiffness of gels produced from PSB and ASRT collagen are not significantly different when compared by compressive mechanical tests, however gels of PSRT collagen are significantly more compliant than both preparations. Using a wound-healing model, contraction of free-floating fibroblast populated collagen lattices, showed gels of PSB collagen contracted significantly more than ASRT collagen. PSB also produced gels containing significantly thinner fibrils than those in equivalent ASRT gels ($p<0.05$; ASRT: 0.159 ± 0.034 μm versus PSB: 0.138 ± 0.033 μm*).* When acid and pepsin collagen from the same source were compared, pepsin solubilisation resulted in significantly more compliant gels, thinner collagen fibrils and a slight reduction in contraction of hydrogels compared to the acid solubilised samples. The differences in stiffness were most likely caused by the cleavage of telopeptides by enzymatic solubilisation, resulting in fewer crosslinking sites and thus thinner collagen fibrils. Applying

biphasic theory to explain the reduction of stiffness of gels containing thinner fibrils is consistent with the observed results. Thinner fibres are less able to support the downward pressure when the load is transferred from the incompressible fluid to the solid collagen network. The source of the tissue also affected the number of crosslinks. Dermal collagen is known to contain more crosslinks naturally as collagen in the dermis is arranged in a basket weave structure whereas collagen in tendons is arranged in parallel strands. Although pepsin cleaves telopeptides, the number of crosslinking sites in the PSB is much higher than in the ASRT or PSRT prior to extraction. The main findings from this section of work suggested that the tissue source i.e. tendon vs. dermis had a more significant effect on the end-product properties than extraction method or species. This could be further investigated by solubilising collagen from tendon and dermis sourced from the same species.

6.1.2 Manipulating collagen properties with ACE Inhibitors

After establishing methods of testing, methods of increasing the gels' stiffness were explored using gels prepared from ASRT collagen. MMP activity is the main cause of degradation of collagen and both the MMP endopeptidases and ACE are enzymes dependent on zinc molecules in their active sites. ACE inhibitors are a group of drugs most commonly used in treating hypertension, however they have been shown to have a significant inhibitory effect on MMPs both *in vitro* and *in vivo.* Thus the starting hypothesis was that inhibiting MMP activity by incorporating ACE inhibitors may reduce or slow degradation of the hydrogels and consequently increase their mechanical stiffness. With a dermal wound healing application in mind, ACE inhibitors also offered attractive properties such as the possibility of

promoting vasodilation in the end product. Three ACE inhibitors: captopril, enalapril and lisinopril were chosen for inclusion, as they represented a wide range of sizes and chemistries from the ACE inhibitor family. Disappointingly, testing the effect of captopril, enalapril and lisinopril on the mechanical properties of collagen hydrogels by measuring bulk stiffness proved inconclusive. Cell viability tests confirmed captopril had a cytostatic effect on embedded cells while enalapril and lisinopril did not affect cell viability. There was no apparent adverse effect on cell number at high initial concentrations of enalapril or lisinopril. When compared with captopril, little of the enalapril and lisinopril were retained in the collagen matrix. This is most likely because of differences in pK_a or size of enalapril and lisinopril, and captopril. Captopril is weakly acidic and small, while enalapril and lisinopril are amphoteric and very large at physiological pH. This could affect the solubility of the drugs, resulting in faster expulsion from the matrix. Improving the retention of the drugs in the gel may increase the effect on their mechanical properties, without adversely affecting viability of the embedded cells and could be further explored in future studies.

Although the inclusion of ACE inhibitors showed some minor improvements in the stiffness, no significant increase in the bulk stiffness of the hydrogels was observed. Other methods of stiffening the hydrogels were therefore explored before settling on a method of increasing bulk stiffness by dehydrating the collagen hydrogels using a new method based on work by Robert Brown and colleagues (Brown et al., 2005)

6.1.3 Controlled dehydration of collagen hydrogels

A simple and cost effective method of dehydration involving simple nylon mesh, filter paper and a super-absorber, sodium polyacrylate, was developed and used to dehydrate gels in situ. This was highly effective in reducing the gel weight by up to 98% within an hour, and thus increasing collagen concentration within the gel 40 fold. After 4 weeks in culture, the number of viable cells in dehydrated gels was not significantly different to those in a hydrated control with cell numbers increasing in both samples. Pro-collagen synthesis increased in dehydrated gels when compared to hydrated gels. Published research has shown that cells with a fibroblastic elongated morphology result in increased expression of new collagen compared to fibroblasts with a spherical cell morphology. Dehydration of the gel promoted adhesion of the embedded cells to the collagen scaffold, resulting in an observed extended morphology consistent with a more fibroblastic morphology compared to the spherical cells observed in hydrated samples. The hypothesis that the cells in the dehydrated gels exhibited a fibroblastic morphology is in turn consistent with the observed increased *de novo* synthesis of pro-collagen in these gels. The observed mechanical properties of the samples also improved dramatically with dehydration. Mechanical testing in tension showed dehydrated gels had Young's moduli of similar magnitude to native tissues. This method could be developed further and has significant promise for the design and manufacture of better scaffolds, such as dermal equivalents.

Supplementing hydrated and dehydrated gels with ascorbic acid or hyaluronic acid individually was investigated but did not affect the cell viability or morphology

significantly and had no effect on the *de novo* synthesis of collagen. However, when the two compounds were added to the gel together, they had a 2-fold effect: improved cell viability and increased *de novo* collagen synthesis. The small sample size of n=4 used in these experiments meant that the statistical tests used were of low power. This suggests that significant differences in the results were either derived from experimental error or from actual differences. Every effort was made to include appropriate controls in each experiment, to reduce the risk of experimental error. Thus, increasing the sample size may show greater significance in these results. As the statistical power of the tests is very low, there is a high probability that there are significant differences in the data which cannot be identified with this sample size.

Scaffolds with elongated cells with accelerated proliferation are highly promising for modelling complex *in vivo* interactions of native ECM. It has previously been reported that cell scaffolds which promote cell adhesion by mimicking native ECM enable the development of fibroblastic morphology and enhance expression of ECM molecules (Pajorova et al., 2018).

6.2 Limitations of this work

6.2.1 Mechanical Testing

Initially, the main aim of this work involved improving the mechanical properties of collagen hydrogels by addition of ACE inhibitors. This necessitated a reliable and sensitive method of measuring the mechanical properties of the hydrogels prior to manipulating them, so that any resultant changes, for example, addition of different

ACE inhibitors, or embedding fibroblasts in the gels, could be measured. Busby's method of confined compression testing with a Bose 3200 was used. Initially, the results were compared with a model developed from biphasic theory to give values for Young's modulus and permeability. This method worked well for the actual testing of the gels, however, post-analysis proved difficult as the regression of the gels' relaxation was used to fit the results to a linear model. The results were inconsistent as the relaxation of the gels was erratic; they often relaxed to less than zero-strain, as the indenter often became stuck to the surface of the gel. This proved problematic and made fitting the results to the biphasic model impossible. A cruder but more repeatable measurement of gel stiffness was subsequently adopted by inferring stiffness from the peak stress value obtained during testing of the gels. This parameter was reliable for diagnosing the relative stiffness of gels within each experiment, however, it limited the translation of the results between experiments.

Soon after starting the experimental phase of the project, the main mechanical testing machine, the Bose 3200, was badly water damaged which prevented access to the machine for several months. The machine was returned to working order and experiments continued, generating the main results of the ACE inhibitor experiments. As part of a departmental reorganisation, the machine was then moved across campus, and the operational amplifier damaged beyond repair. This necessitated continuing experiments of a different test rig. A smaller compression-testing rig was developed with a Bose 3100. After beginning experiments with the Bose 3100, it was discovered that the Bose 3100 and 3200 had not been calibrated for several years, resulting in vastly different readings between control gels tested in

each machine. This unavoidably introduced a large source of potential systematic error into the mechanical testing results, as neither of the machines could be trusted to give accurate readings in absolute terms. With only the Bose 3100 available for use, and the earlier experiments being unrepeatable due to the destructive nature of the testing, a set of control gels were prepared and tested on the Bose 3100 in exactly the same way as had been done on the Bose 3200 to compensate, as far as possible, for the systematic errors. It was assumed for the purpose of the study that the control gels prepared in the same way and tested at the same time points on each machine would have the same stiffness, and this hypothesis was supported by the repeatable nature of the results obtained from both machines. Subsequently, the results of tests of gels supplemented with ACE inhibitors, or acetylcysteine and collagenase inhibitor were normalised to the results obtained for control gels tested in the respective machines to allow comparison of the initial and subsequent experimental findings.

It was also found that the result of mechanical testing in compression was severely affected by surface seeded cells. Collagen hydrogels are a biphasic material comprising a liquid phase of fluid trapped within a solid network of collagen fibrils. When collagen hydrogels are tested in confined compression with a porous platen, the incompressible fluid phase initially bears the load while it remains trapped within the network. As the fluid begins to be displaced through the porous platen, the solid network of collagen fibrils bears the load. A confluent layer of cells on the upper surface of the collagen gel further traps the fluid within the hydrogel. As fluid is incompressible, and a confluent cell layer means it has nowhere to escape to, this can

give falsely high readings of stiffness of the underlying collagen gel when tested in confined compression. After identifying this, hydrogels were prepared with cells embedded throughout the collagen scaffold rather than on the surface of the scaffold. This improved the repeatability of the mechanical testing method as evidenced by reduced standard deviations in the results obtained between samples.

A further method of mechanical testing was developed to mechanically characterise hydrated and dehydrated collagen gels in tension. In practice the viability of this method was limited by the multiple handlings of the gels to manoeuvre them into the testing rig and subsequently into the testing machine. Many of the gels were damaged even before testing so results were unreliable. Additionally, a small load cell and a maximum extension of 4 mm proved to be insufficient to reach the breaking point of those gel samples undamaged by the handling process, and for some of the samples was barely enough to extend the gels beyond their elastic region. Either designing new casting plates and a tensioning device for use with the Bose 3100 or finding a larger mechanical testing machine for use with the casting plates and testing rig currently available would be required to fully test the hydrogels in tension reliably.

In general, the value of the conclusions from the mechanical testing experiments was limited by the lack of reliability and the inherent limitations of the available equipment. An alternative method, such as rheological testing, a common method used in testing viscous fluids, could be used to test the bulk mechanical properties of the hydrogels. This could be useful in overcoming the limitations of the testing

methods used in this work. Testing the strength or stiffness of individual collagen fibrils could be used to test mechanical properties on a cellular scale. This could have been accomplished by AFM, however, this was not possible in the time available.

6.2.2 Fluorescence microscopy

The fluorescent microscope within the department (Zeiss upright Axioscope model with attached Apotome) used to examine the morphology of HDFs in hydrated and dehydrated collagen gels unexpectedly malfunctioned during examination of the 4-week cell culture samples, with the result that two different microscopes had to be used. The 1-week samples were all examined with the Biomedical Engineering department's fluorescent microscope mentioned above, which optically sectioned the samples with an Apotome, while 4-week samples were examined with a confocal microscope (Leica model SP8) located in an adjacent building in SIPBS. This brought further variation, as field of vision, image quality and range were very different between the two microscope types. Ideally, the samples from both time points would be re-examined with the same type of microscopy, however, due to time limitations, this was not possible.

6.2.3 Collagen Types

Whilst comparing collagen types, only 3 types of collagen were available: pepsin soluble bovine collagen, pepsin soluble rat-tail collagen and acid soluble rat-tail collagen. However, for the isolation of the pepsin solubilised collagens, both the species (rat versus cow) and the tissue from which it was isolated (tail tendon versus dermis) were different. This introduced a number of variables potentially affecting

the mechanical properties of the resulting gels and made it difficult to pinpoint exactly what led to differences in observed properties between the different gels. To elucidate whether species or tissue had a greater effect, collagen isolated from rat dermis or bovine tendon would have been useful to determine whether tissue or species has more influence over the properties of the extracted collagen. Equally, sourcing acid soluble bovine collagen would be useful to compare the full range of variables of collagen preparation types and sources.

6.3 Further work and Applications

6.3.1 Mechanical testing

The major limitation of the current work was the inability to repeat the measurements of stiffness and the unreliability of the machines. Developing a new, reliable quick method of mechanical testing is critical going forward both to improve repeatability and to increase the sensitivity of the measurements. Currently, mechanical testing in confined compression is a destructive process as it is not sterile. Repeated testing of the same sample, especially when seeded with cells, brings its own problems, however, it may reduce the sample number required. Ideally, two approaches to mechanical testing should be undertaken: an experimental method to measure bulk mechanical properties of the overall hydrogel; and an approach to measure the mechanical properties on a cellular scale to more accurately measure the forces cells are subjected to within the scaffold.

6.3.2 Comparing different collagen preparations

Collagen is vastly different in native tissue depending on which tissue it is located in, and the age and species of the animal. Collagen in skin is inherently different from collagen in tendon, as in skin it is laid out in a basket weave and heavily cross-linked compared to tendons (Berthod et al., 2001). In tendons and muscle, collagen exists in parallel bundles, in line with the direction of mechanical stress (Svensson et al., 2017) resulting in significantly fewer crosslinking sites. Equally, the age and species of the animal from which the collagen has been obtained will also affect the collagen structures; an older animal will have more crosslinks in its collagen structure than a younger animal (Craig et al., 1989).

Comparing collagen solubilised with acid from rat-tail tendons and collagen solubilised with pepsin from bovine dermis was very useful as these are the main preparations of collagen used for laboratory and medical applications, respectively. They were shown to be very similar in terms of stiffness, contraction profile when seeded with fibroblasts and fibril formation (Section 3.3). When collagen solubilised with pepsin from rat tail tendons was compared to the two other preparations, it was much more compliant, had a very different contraction profile when contracted with fibroblasts and had much thinner and less defined fibrils. It was inconclusive as to whether these differences arose from the variation in species or in tissue. The difference in contraction, however, did not appear to arise from preparation method. Examining collagen from the same tissue source and different species, or the same species and different tissue source would allow us to conclude what the reasons for these differences were. Fully understanding how species, age and tissue affects

collagen makeup would reduce variation in the properties of preparations and could also be useful in providing knowledge on new sources of collagen. Conversely, a greater understanding of how the collagen source affected the final product could allow manipulation of the behaviour of the collagen by choosing an appropriate source tissue, i.e. species, tissue and age of animal.

6.3.3 ACE inhibitors

There is still much work to be carried out on the effects of ACE inhibitors in relation to collagen scaffolds. Neither the effect, nor the mechanism behind the effect is fully understood. A different approach to that taken in this study is essential to fully understand the underlying issues. For example, collagen zymography should be studied with collagen as a substrate for a variety of MMPs and collagenases. This would categorically confirm or reject the theory that the ACE inhibitors are affecting the mechanical properties of collagen scaffolds by inhibiting degradation of the scaffold, thus maintaining the integrity of the hydrogel.

To test whether ACE inhibitors directly inhibited collagenase, an SDS-PAGE study was briefly undertaken. Collagen gels, supplemented with ACE inhibitors, were incubated with type II collagenase. The products of the digestion were tested by SDS-PAGE. The collagen samples were completely digested and there was no difference in the digestion results from control gels and those containing ACE inhibitors. After the experiments were completed, it was noticed that when collagen scaffolds containing ACE inhibitors were digested with collagenase to release cells for the MTT assay, the gels containing ACE inhibitors took much longer to be

digested than their control gel counterparts, consistent with the hypothesis that the ACE inhibitors were inhibiting degradation of the collagen scaffold by the collagenase. However, this was not shown by the SDS-PAGE studies. A wider range of concentrations of collagenases and ACE inhibitor could be tested and the SDS-PAGE tests repeated at different time-points to measure if the degradation of the collagen gel, by collagenase, was slowed by the addition of ACE inhibitors. Alternatively, quantitative measurements of collagen degradation products in the media of gels cultured with fibroblasts and ACE inhibitors could be carried out to test whether the addition of cells affected the ACE inhibitor effect on collagenase. A new, more reliable mechanical testing process would allow mechanical tests to be extended to include a wider range of concentrations of ACE inhibitors and time points to fully understand what effect the ACE inhibitors are having on the gel stiffness over time. Alternatively, ACE inhibitors could be added to the gel after setting, rather than adding ACE inhibitors to the hydrogel mixture prior to setting. This could help to establish whether the ACE inhibitors cause their effect by bringing about a physical change, for example, by being trapped in the network, or whether the effect is chemical.

6.3.4 Dehydration of Collagen hydrogels

The method presented in this thesis delivers rapid dehydration of collagen hydrogels whilst maintaining viable cells in the scaffold. Dehydration with 10% w/w sodium polyacrylate led to 96 % weight loss after 10 minutes and 98% weight loss after 1 hour, resulting in collagen concentrations comparable to native tissues. This was a very successful outcome. In refining the scaffold for a dermal application, hyaluronic

acid and ascorbic acid were included in the matrix because of their high concentrations and important roles in human skin. When added to the gels together, these chemicals had a positive effect on cell viability, morphology and pro-collagen synthesis. The range of GAGs included in the collagen scaffolds could be extended with a view to improving or manipulating the biocompatibility of the dehydrated gel further. Additionally, the work reported in chapter 5 suggests adding enalapril or lisinopril could be used to improve the performance of the cell scaffolds in terms of blood flow, collagen synthesis, inhibiting degradation of the scaffold and assisting in wound healing. Captopril should be avoided as it was found to have a cytostatic effect on fibroblasts *in vitro*, suggesting it would have a similar cytostatic effect *in vivo.*

After 4 weeks, fluorescent microscopy showed that the collagen scaffold retained the reverse pattern of the nylon mesh used during dehydration (Figure 5.13; 168). Topography and stiffness have been well reported as guides for cell morphology and behaviour, especially of keratinocytes and mesenchymal stem cells (Bernstam et al., 1990; Fu et al., 2014; Abagnale et al., 2015). Patterning of collagen scaffolds could be exploited to improve viability of a cell co-culture. A co-culture of stratified keratinocytes would dramatically increase the effectiveness of these scaffolds as skin grafts (Compton et al., 1998). Further tests could be undertaken to test the dehydrated hydrogels as cell scaffolds. An air interface experiment could be prepared, with a dehydrated gel containing fibroblasts and surface seeded with keratinocytes. This would be an excellent method to test the performance of

dehydrated scaffolds to simulate skin grafts (Bernstam et al., 1990; Boyce et al., 2002).

6.4 Application and translation of this work

6.4.1 ACE Inhibitors

Although much of the work relating the effect of including ACE inhibitors in cell embedded scaffolds on their mechanical properties proved inconclusive as to whether their inclusion increased bulk stiffness, the studies of the effect of the inclusion of ACE inhibitors on the viability of embedded cells carried out alongside the mechanical testing could prove useful in improving the design of cell scaffolds for wound dressing applications. There is a substantial body of published research showing ACE inhibitors can stimulate *de novo* collagen synthesis (Claridge et al., 2004; Ihm et al., 1994) and can reduce degradation of collagen *in vivo* (Brower et al., 2007; Levick et al., 2007; Sakata et al., 2004; Ihm et al., 1994; Sorbi et al., 1993) in a variety of morbidities (Robertson, 1994; Herman and Bhimji, 2018; Duchin et al., 1982; Hansson et al., 1999; Claridge et al., 2004; Brower et al., 2007; McElmurray et al., 1999). The incorporation of enalapril and lisinopril did not adversely affect cell viability in the *in vitro* experiments reported here. Use of ACE inhibitors in wound dressings could therefore have several positive actions:

(i) increasing blood-flow to the site of injury to aid healing

(ii) dilating the surrounding blood vessels whilst improving the rate of healing

(iii) increasing *de novo* collagen synthesis and

(iv) decreasing the rate of degradation of collagen already present in the wound.

A more focussed study *in vitro* and *in vivo* on the local application of ACE inhibitors in healing wounds would be useful in establishing whether there are any benefits from this treatment. The benefits of a collagen scaffold in wound treatment have been well established and there are a number of wound treatments and dermal equivalents currently available as outlined in Table 1.3 (Page 25), as well as drug releasing biomaterials available for wound treatment (Johnson and Wang, 2015) and cell based biomaterial treatments (Pourmoussa et al., 2016). Depending on the outcomes of such in vitro and *in vivo studies*, incorporating ACE inhibitors into established collagen dermal scaffolds could be valuable in improving wound treatments.

Although the ACE inhibitors didn't dramatically increase the stiffness of the hydrogels as hoped, published research suggests there would be other benefits from enhanced blood flow to the area and increased collagen deposition. Rather than trying to overcome the mechanical instability of collagen hydrogels, alternative preparation methods could be employed. Matthews and colleagues (Matthews et al., 2002) established a method of electrospinning collagen fibres which produced scaffolds with tuneable mechanical properties of any shape. These scaffolds retained collagen fibres with the native collagen banding patterns which encouraged cell infiltration. Hall-barrientos and colleagues subsequently conjugated electrospun collagen fibres with the antibiotics levofloxacin and irgasan (Hall Barrientos et al., 2017) which had a controlled release over time. Combining a collagen based biomaterial with ACE inhibitor or alternative drugs or co-factors could be useful.

6.4.2 Dehydration of collagen hydrogels

Dehydration of collagen hydrogels with sodium polyacrylate proved extremely successful. The concentration of collagen increased in the hydrogels to almost 40 times that of fully hydrated gels with 10% w/w SP. This gave a hydrogel with a collagen concentration of around 12% and a mechanical stiffness of 6.34 kPa (without cells) which approached that of native skin tissue, which is reported to have a stiffness of 8.2 kPa in tension (Zahouani et al., 2009) and a collagen concentration of 70% of its dry weight (approximately 15-20% of wet weight depending on skin hydration, age and location). As well as improving concentration and mechanical stiffness, the dehydration process supported viable human dermal fibroblasts within the hydrogel, which were shown to be viable up to 4 weeks. Although the RAFT™ system also has some of these benefits, such as supporting cells and improving stiffness, the dehydration method involving sodium polyacrylate, outlined in this work, produces the same, and more, benefits e.g. improved cell viability and higher collagen concentrations, at a fraction of the cost. At time of publication, this method gave a cost saving of over 99% compared to the RAFT™ system.

The sodium polyacrylate method also increases the flexibility of the technique, as any size or shape of hydrogel can be dehydrated, as long as a suitable casting plate is available. This method also reduced handling of the final product, decreasing the likelihood of mechanical damage of the final product compared to the RAFT™ system. The reduced handling requirements also reduce the risk of compromising the sterility of the hydrogel during handling and transfer of the product when compared to the method from Brown's laboratory. Our early experiments suggest topography is

stable in the hydrogel for at least 4 weeks. This could be further explored as a means of improving viability of co-culture, for cultivating layers of differentiated keratinocytes and fibroblasts to maintain more complex skin models. These would provide valuable skin graft materials for the treatment of burns patients, and experimental models for use in in vitro toxicity testing of skin products for the pharmaceutical and cosmetics industries.

Beyond skin-based applications, there are several other tissue-engineering applications which could benefit from this work. The technique showed that including hyaluronic acid and ascorbic acid in the hydrogel improved cell morphology, collagen synthesis and cell viability. This may have been caused by a macromolecular crowding phenomenon. Concentrating large molecules such as GAGs, fibronectin or laminin can be easily achieved while retaining the native architecture of the collagen structure through this method. The biochemistry of scaffolds can be easily tailored to an environment suited to bone, cardiac, or liver tissue for example. As well as this, the dehydration process can be easily controlled to manipulate the mechanical strength and modulate the concentration of collagen and other molecules in the scaffold.

Controlling the surface topography through the dehydration process is also an avenue for future investigation; this dehydration technique showed that the scaffold retained a stable surface topography imprinted during dehydration. Mesenchymal stem cells can be differentiated by controlling the surface topography of the cell substrate (Dalby et al., 2007; Abagnale et al., 2015; Casanellas et al., 2019; Cun and Hosta-

Rigau, 2020). Surface topography can also act as a cue for cell proliferation and adhesion for cells. Controlling the surface topography using this technique could be useful for a variety of tissue engineering applications, but would be ideal in creating cartilage tissue specifically. Cartilage contains a high concentration of collagen and GAGs, is not vascularised and contains chondrocytes. The possibilities for this technique are numerous, other applications such as drug delivery (Albu et al., 2011; Li and Mooney, 2016) or encapsulating biosensors (Ju et al., 2009) are also prime candidates for investigation.

While the work completed in this thesis has perhaps left more open questions than answers, it has also led to a whole series of further studies that I would like to see tackled. Early experiments with this dehydration system looked extremely hopeful and could prove invaluable to the tissue engineering community if developed.

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Appendix

Statistical Analysis of Chapter 5:

Controlled Dehydration of collagen hydrogels

i Statistical Analysis of Chapter 5: Controlled Dehydration of collagen hydrogels

i.i Dehydration of collagen gels without cells

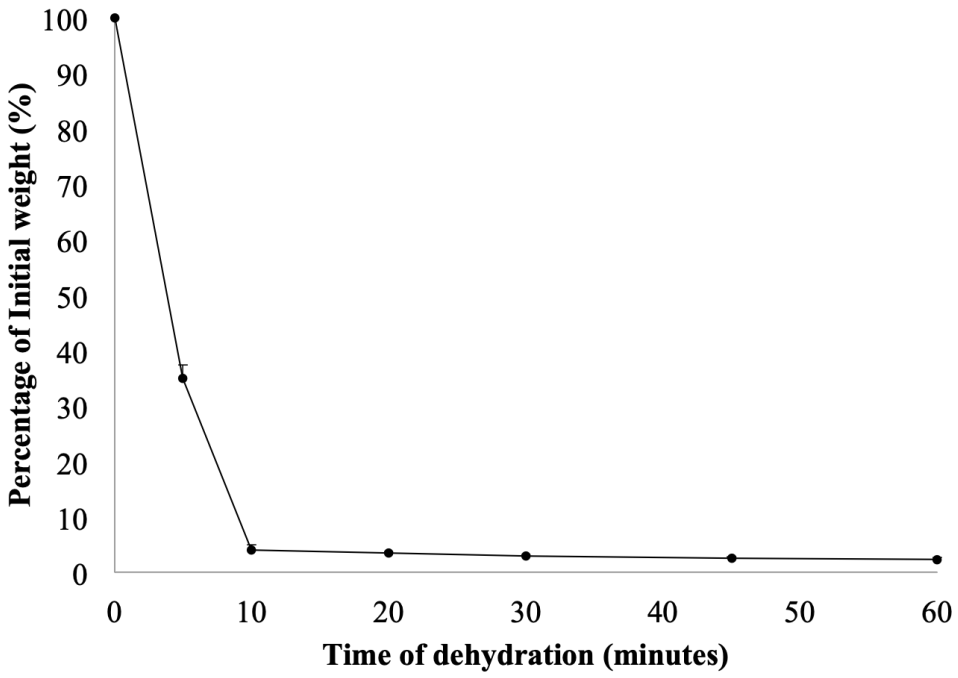


Figure i.i: Percentage of initial weight after dehydration with sodium polyacrylate (10% w/w) for varying times. n=4, mean + SEM. Standard error within marker at 20, 30, 45 and 60 minutes.

Collagen hydrogels initially containing 0.3% (w/v) rat-tail collagen were dehydrated with 10% (w/w) sodium polyacrylate (SP) for 5, 10, 20, 30, 45 or 60 minutes. The percentage of initial weight remaining after each dehydration time is presented in Figure i.i. The results were compared by a one-way ANOVA followed by post-hoc analysis with a Tukey's test, with each time point compared to the other time points (Table i.i). All dehydrated samples were significantly different ($p<0.0001$) to the hydrated sample. There was no significant difference between samples dehydrated for 10, 20, 30, 45 or 60 minute ($p>0.05$).

Table i.i: Results of one-way ANOVA of percentage of gel weight remaining after dehydration for 0, 5, 10, 20, 30, 45 or 60 minutes with 10% (w/w) sodium polyacrylate followed by a post-hoc Tukey's test. Confidence level 95%.

	0 m	5 m	10 m	20 m	30 m	45 m	60 m
0 m		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
5 m			<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
10 m				>0.05	>0.05	>0.05	>0.05
20 m					>0.05	>0.05	>0.05
30 m						>0.05	>0.05
45 m							>0.05
60 m							

i.ii Viability of hydrated and dehydrated collagen hydrogels as long and short-term scaffolds for human dermal fibroblasts

To measure cell viability, an MTT assay was carried out on a sectioned area of the gel. As MTT is a colorimetric viability assay for cell metabolism, the absorbance was compared to a standard curve of number of human dermal fibroblasts against absorbance of MTT formazan formed. The MTT assay showed viable cells in both hydrated and dehydrated gels after 1 and 4 weeks (Figure i.ii). As can be seen, there was a significant increase in cell number in both gel preparations from initial seeding to 1 week and from 1 week to 4 weeks suggesting cells were dividing. When statistically compared by one-way ANOVA, there was a transient significant increase in cell numbers between hydrated and dehydrated gels after 1 week *($p<0.05$)*. However, after incubation for four weeks there was no significant difference between the hydrated and dehydrated gels *($p=0.35$)*. There was a significant increase in cell number in both preparations from 1 week to 4 weeks in culture (*$p<0.01$*).

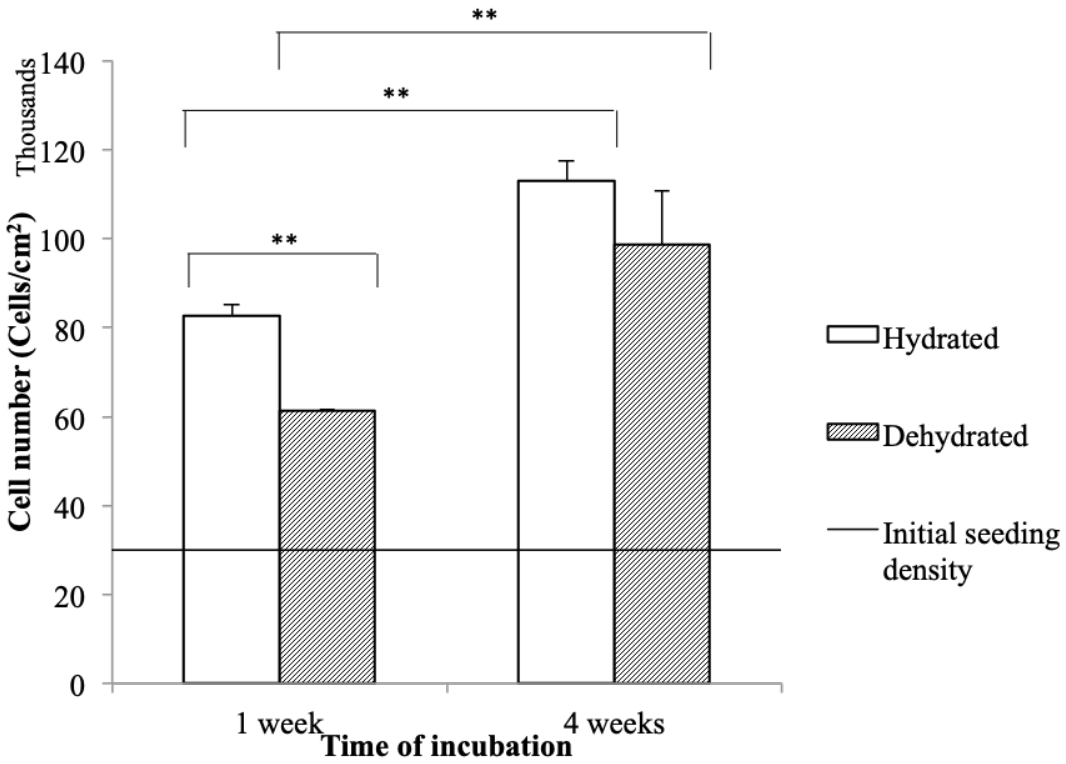


Figure i.ii: Viability of HDFs measured by MTT after 1 or 4 weeks in culture. Collagen hydrogels (0.3% w/v) were prepared with HDFs then dehydrated with sodium polyacrylate (10% w/w). The solid line signifies initial seeding density (30,000 cells/cm^2). n=4, mean + SEM. A one-way ANOVA was used to compare the mean of each group. * indicates *p<0.05, ** p<0.01.* There was no significant difference between 4 week hydrated and dehydrated gel (*p=0.*35).

An ELISA for pro-collagen I was used to assess whether cells were producing *de novo* extracellular matrix (Figure i.iii). The ELISA was completed on media changes from 1 or 4 weeks in culture. There was no significant difference in pro-collagen concentration produced in hydrated and dehydrated gels after 1 week. There was a significant increase in pro-collagen concentration from HDFs in dehydrated gels from 1 week to 4 weeks (*p<001*).

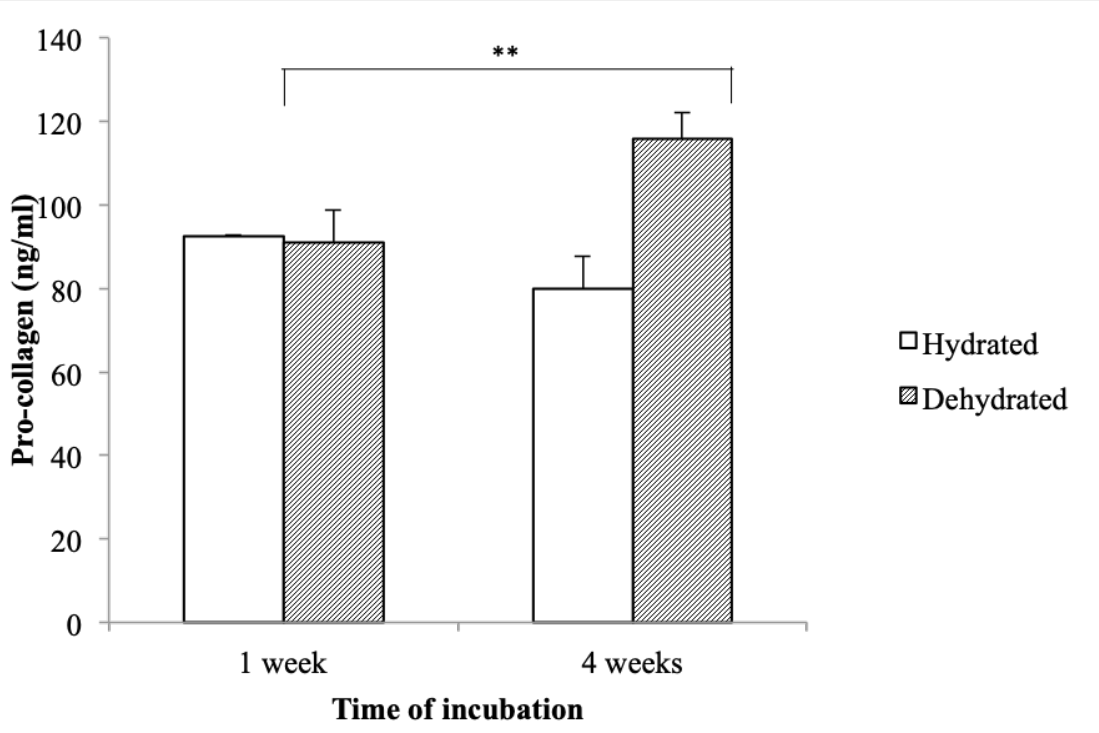


Figure i.iii: Concentration of pro-collagen concentration produced by HDFs measured by ELISA. Collagen hydrogels (0.3% w/v) prepared with HDFs then dehydrated with SP (10% w/w). Concentration of pro-collagen measured in media changes by ELISA. n=4, mean + SEM. A one-way ANOVA was used to compare the mean of each group. * indicates *p<0.05, ** p<0.01.*

i.iii Supplementing hydrogels with Ascorbic Acid

To improve cell viability and increase synthesis of *de novo* collagen in scaffolds, ascorbic acid was added to the gel scaffolds. Concentrations of AA from 0 to 50 μg/ml were added to cell culture medium prior to adding to the cells. Cell viability was measured by MTT (Figure i.iv). The concentration 17.6 μg/ml, equivalent to 100 μM, showed the greatest increase in cell viability, with greater concentrations beginning to inhibit the viability.

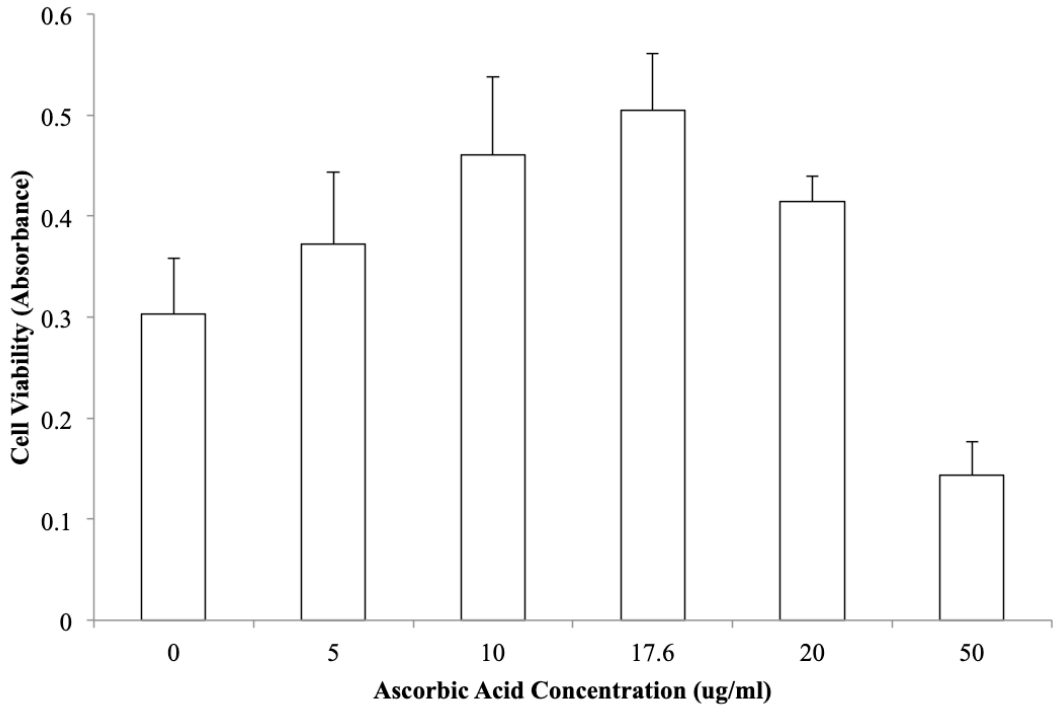


Figure i.iv: Viability of Human Dermal Fibroblasts, measured by MTT, when supplemented with concentrations of 0 to 50 µg/ml of ascorbic acid. n=4, mean + SEM.

i.iv Viability of hydrated and dehydrated collagen hydrogels supplemented with ascorbic and hyaluronic acid as long and short-term scaffolds for human dermal fibroblasts

To assess the viability of hydrated and dehydrated collagen hydrogels for the long and short-term culture of HDFs, collagen gels were prepared in a number of ways (Table i.ii). Hydrated and dehydrated collagen gels prepared without any additive, with ascorbic acid, with hyaluronic acid and with both ascorbic and hyaluronic acid were maintained for 1 or 4 weeks in culture. HDFs were added to the hydrogels prior to setting, at a density of 3×10^4 cells/cm^2. A set of cell-free controls was also prepared. Collagen gel weight, cell viability, collagen degradation, new collagen synthesis and cell morphology were all studied after 1 or 4 weeks.

Table i.ii: Collagen hydrogels prepared for long and short-term support of HDFs. Collagen gels were prepared with or without human dermal fibroblasts and additives then left hydrated or dehydrated with SP. Each group maintained for 1 or 4 weeks, n=4.

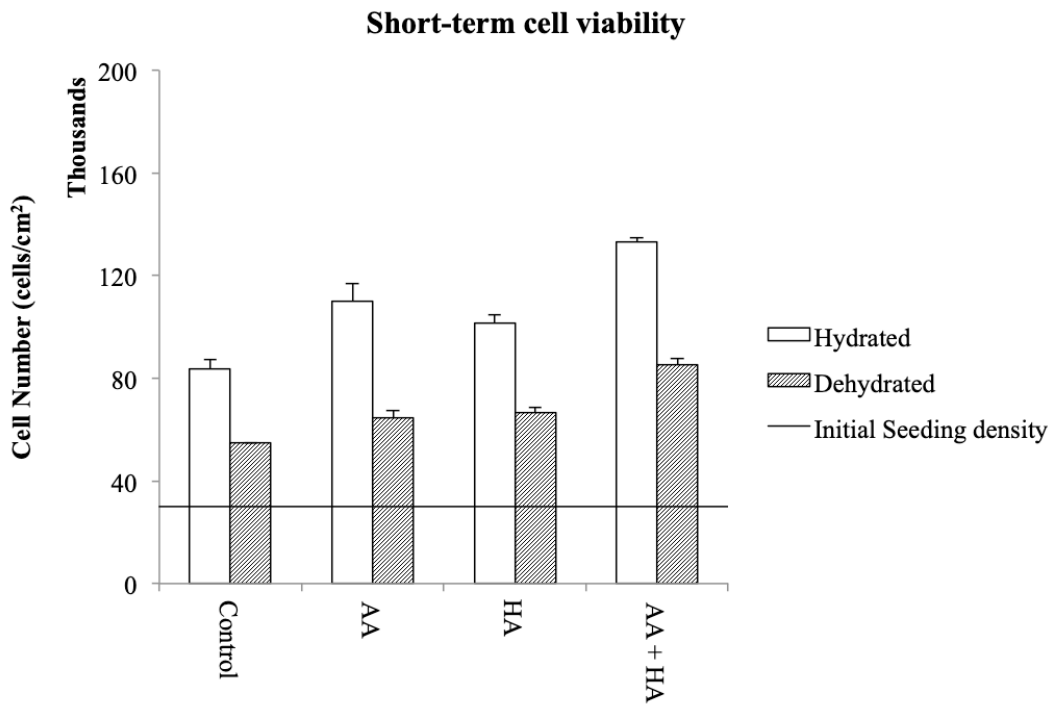
Dehydrated	Collagen	Collagen + AA	Collagen + HA	Collagen + AA + HA	+ HDFs
	Collagen	Collagen + AA	Collagen + HA	Collagen + AA + HA	- HDFs
Hydrated	Collagen	Collagen + AA	Collagen + HA	Collagen + AA + HA	+ HDFs
	Collagen	Collagen + AA	Collagen + HA	Collagen +AA +HA	- HDFs

i.iv.i Cell viability in dehydrated collagen gels

The number of metabolising HDF cells was measured after 1 or 4 weeks in hydrated and dehydrated collagen scaffolds (Figure i.v). Collagen scaffolds were initially seeded with $3x10^4$ HDFs/cm^2. After 1 week (Figure i.v (a)), cell number had increased significantly in all gels compared to the initial seeding level. Cell numbers in both hydrated and dehydrated hydrogels followed a very similar pattern with gels supplemented with AA displaying increased cell numbers compared to control gels and gels supplemented with HA while gels supplemented with both AA and HA showed the greatest cell viability. Dehydrated gels contained fewer cells than hydrated gels overall. In the longer term (Figure i.v (b)), cell numbers had recovered in control gels when comparing hydrated and dehydrated gels. Gels supplemented with HA alone had a detrimental effect on cell number, while supplementing the

hydrogels with AA seemed to improve cell viability. After 4 weeks, cell number remained greatest in gels supplemented with both AA and HA.

(a)



(b)

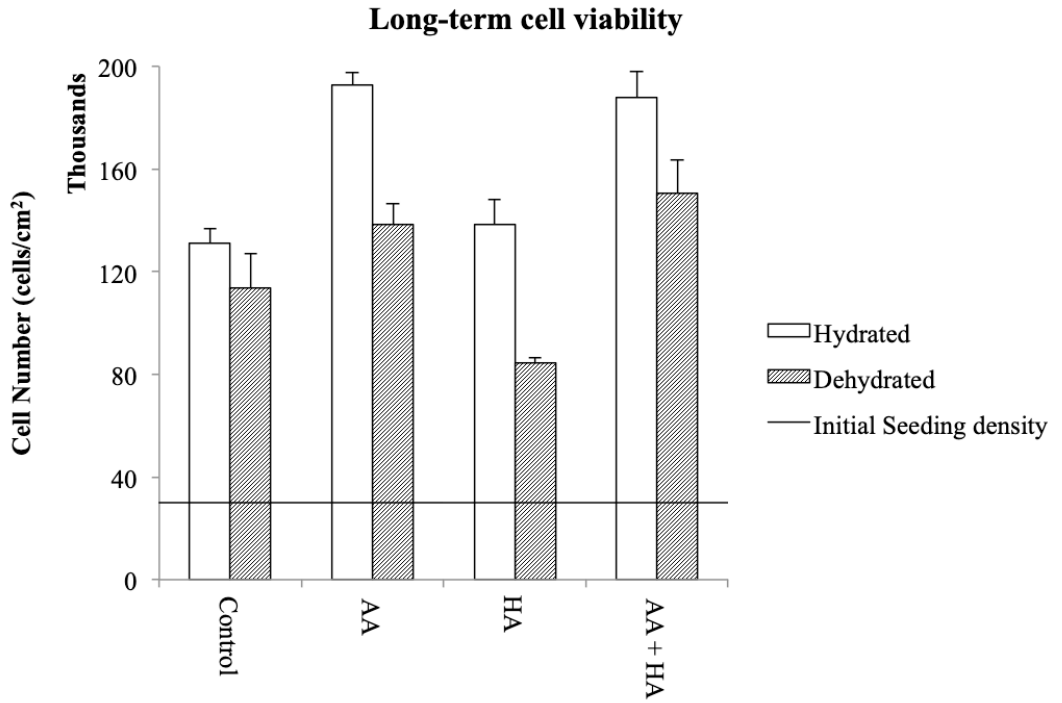


Figure i.v: Cell viability in hydrated and dehydrated collagen hydrogels measured by MTT assay after (a) 1 week or (b) 4 weeks in culture. Gels were prepared with Human Dermal Fibroblasts then supplemented with: ascorbic acid (AA); hyaluronic acid (HA) or both. n=4, mean + SEM.

A statistical analysis was carried out in which a single variable: cell number, was compared with several variable factors by the general linear model. Four factors were considered: time in culture, initial cell seeding, hydration state and type of gel additive; as well as their interaction terms (Table i.iii). Each individual factor had a significant effect on cell number ($p<0.001$). The interaction of two factors were also tested, all interactions were significant ($p<0.001$) except the interactions between time and hydration and between type of additive and hydration. Neither three factor interactions nor four factor interactions had a significant impact on cell number ($p>0.05$).

Table i.iii: Results of statistical analysis of cell number compared by the general linear model including four factors:time in culture; initial cell seeding; gel additive and gel hydration.

Tests of Between-Subjects Effects

Dependent Variable: Cell Number

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	9.874[a]	31	.319	145.397	.000
Intercept	10.073	1	10.073	4597.848	.000
Time	.543	1	.543	247.795	.000
Cells	7.585	1	7.585	3462.448	.000
Additive	.127	3	.042	19.294	.000
Hydration	.358	1	.358	163.611	.000
Time * Cells	.448	1	.448	204.288	.000
Time * Additive	.047	3	.016	7.138	.000
Time * Hydration	.000	1	.000	.103	.749
Cells * Additive	.215	3	.072	32.679	.000
Cells * Hydration	.185	1	.185	84.555	.000
Additive * Hydration	.009	3	.003	1.353	.259
Time * Cells * Additive	.040	3	.013	6.144	.001
Time * Cells * Hydration	.002	1	.002	.696	.405
Time * Additive * Hydration	.013	3	.004	1.927	.127
Cells * Additive * Hydration	.011	3	.004	1.731	.162
Time * Cells * Additive * Hydration	.008	3	.003	1.192	.314
Error	.386	176	.002		
Total	21.211	208			
Corrected Total	10.260	207			

a. R Squared = .962 (Adjusted R Squared = .956)

There are limitations in applying the general linear model to this experimental data, primarily from attempting to ascertain the effect of a number of independent variables on the cell numbers from a small number of samples, as the uncertainty in the tests

increases inversely with the sample size. Although over 300 samples were prepared and over 300 measurements of cell number carried out, along with measurements of sample weight, pro-collagen synthesis and microscopy on each sample, this equated to only four samples for each combination of independent variable. Increasing the number of samples would however decrease uncertainty as to how accurate test is. To reduce the complexity of the testing, and provide more useful statistical analysis, various hypotheses were tested by one-way ANOVA. This presents its own problems, as sub-sections of the data are treated as independent data sets without looking at it as part of the whole picture.

i.iv.i.i Hypothesis testing by one-way ANOVA

Cell numbers were compared by one-way ANOVA followed by a post-hoc analysis Dunnett's test to compare the mean cell number of each group to the mean of the control group.

After 1 week, do any of the additives have a significant effect on cell number in dehydrated gels?

The cell numbers present in dehydrated hydrogels after 1 week were compared by a one-way ANOVA followed by a post-hoc Dunnett's test. The results are presented in Table i.iv. Ascorbic acid alone did not increase the cell number significantly while both Hyaluronic acid and Hyaluronic acid in combination with Ascorbic acid increased the number of cells significantly ($p<0.001$).

Table i.iv: Results of post-hoc analysis of a one-way ANOVA with a Dunnett's test. The number of HDF's contained in dehydrated collagen gels after 1 week supplemented with HA, AA or both, were compared to dehydrated controls.

(I) Additive	(J) Additive	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
AA	Control	.02343333	.01196583	.147	-.0066348	.0535015
HA	Control	.05485000*	.01126545	.000	.0265418	.0831582
AA+HA	Control	.09743333*	.01196583	.000	.0673652	.1275015

After 4 weeks, do any of the additives have a significant effect on cell number in dehydrated gels?

After 4 weeks, HA nor AA significantly changed the number of cells in dehydrated gels while AA and HA in combination increased the cell number significantly compared to the control group ($p<0.05$).

Table i.v: Results of post-hoc analysis of a one-way ANOVA with a Dunnett's test. The number of HDF's 1 week in culture supplemented with HA, AA or both, were compared to dehydrated controls.

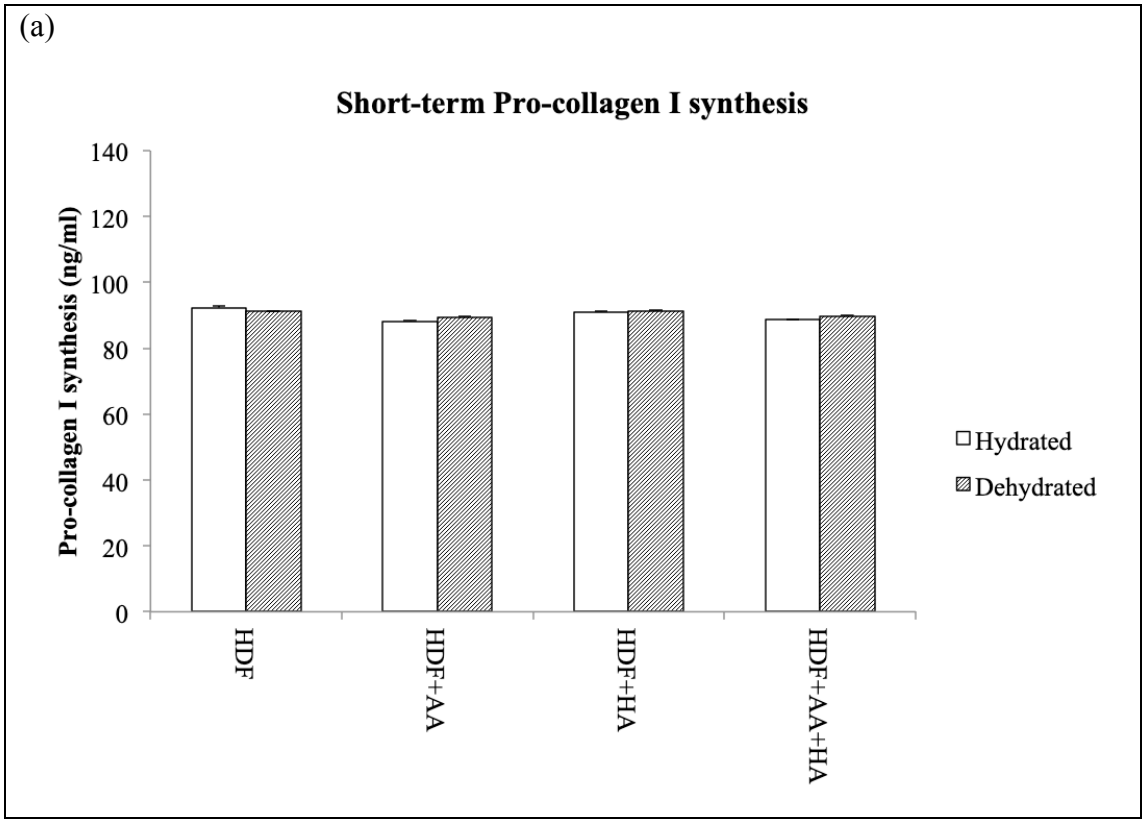
(I) Addition	(J) Addition	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
AA	Control	.07891667	.05041267	.287	-.0468407	.2046741
HA	Control	-.09190476	.05193299	.204	-.2214547	.0376452
AA+HA	Control	.13138095*	.05193299	.046	.0018310	.2609309

i.iv.ii Generation of new collagen by embedded fibroblasts

Pro-collagen, measured by ELISA, was used as a proxy to quantify *de novo* collagen synthesis in the samples (Figure i.vi). Each media change was collected and frozen until the end of the experiment. The frozen media was mixed, defrosted and centrifuged; then a sample tested to measure the concentration of pro-collagen present. After 1 week (Figure i.vi (a)), all gels containing HDFs were found to be producing pro-collagen at a concentration of approximately 90 ng/ml of spent media. By 4 weeks in culture (Figure i.vi (b)), concentration of pro-collagen in control gels was significantly higher in the dehydrated gels (115.9 ± 6.3 ng/ml) compared to hydrated gels (68.6 ± 2.0 ng/ml). In hydrated gels, synthesis was greater in those gels supplemented with ascorbic acid, and hyaluronic acid. Ascorbic acid seemed to have the greatest impact, as supplementing with this alone increased synthesis to 100.2 ± 11.5 ng/ml, while hyaluronic acid alone increased synthesis to 85.0 ± 12.2 ng/ml. When hydrated gels were supplemented with both hyaluronic acid and ascorbic acid, collagen synthesis increased to 120.4 ± 1.3 ng/ml. The results for dehydrated collagen gels differed from those for hydrated gels as the unsupplemented control gels produced more de novo collagen than supplemented gels. Unsupplemented dehydrated collagen gels produced 115.9 ± 6.3 ng/ml of de novo

collagen. Where ascorbic acid promoted synthesis in hydrated gels, it inhibited synthesis in dehydrated gels, gels supplemented with ascorbic acid producing 90.8 ± 6.8 ng/ml when added alone and 87.9 ± 2.2 ng/ml when added along with hyaluronic acid. Compared to ascorbic acid, hyaluronic acid inhibited synthesis less, giving a concentration of 94.9 ± 4.1 ng/ml.

(a)



(b)

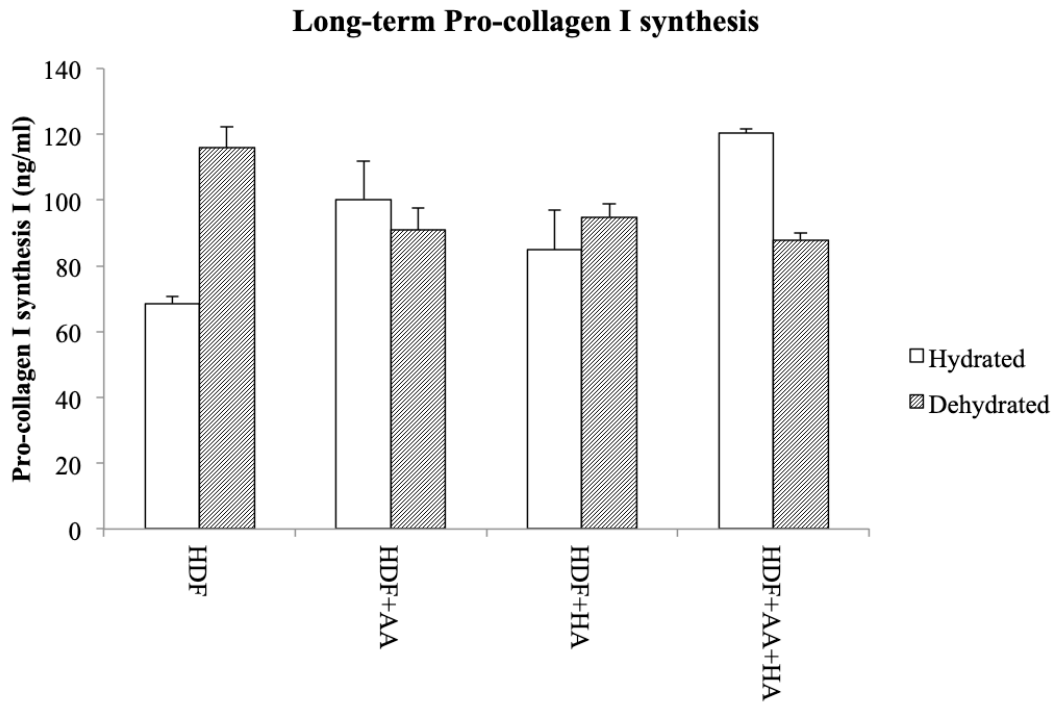


Figure i.vi: Concentration of newly synthesised collagen present in media changes after (a) 1 week or (b) 4 weeks, measured by pro-collagen I ELISA. Gels were prepared with Human Dermal Fibroblasts (HDF) then supplemented with: ascorbic acid (AA); hyaluronic acid (HA) or both. n=4, mean + SEM.

The quantity of collagen synthesis products were statistically compared by the general linear model. A dependent variable: the quantity of pro-collagen, was compared with 3 variable factors: time in culture, hydration of the gel and additives added to the gel. As well as the interaction of each of these factors. Results are displayed in Table i.vi.

Table i.vi: Statistical analysis by the general linear model of quantity of pro-collagen synthesised by HDFs in collagen gels and the effect of time in culture, additive to the gel and hydration of the gel.

Tests of Between-Subjects Effects

Dependent Variable: Collagen Synthesis

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	7967255234.733[a]	11	724295930.430	3.064	.002
Intercept	688080806976.167	1	688080806976.167	2910.618	.000
Time	373953164.138	1	373953164.138	1.582	.212
Hydration	1777067.308	1	1777067.308	.008	.931
Additive	2718987414.367	3	906329138.122	3.834	.013
Time * Hydration	.000	0	.	.	.
Time * Additive	4346530830.290	3	1448843610.097	6.129	.001
Hydration * Additive	14306887.489	3	4768962.496	.020	.996
Time * Hydration * Additive	.000	0	.	.	.
Error	18203081722.932	77	236403658.739		
Total	772171749188.018	89			
Corrected Total	26170336957.665	88			

i.iv.ii.i Hypothesis testing by one-way ANOVA

Cell numbers were compared by one-way ANOVA followed by a post-hoc analysis Dunnett's test to compare the mean cell number of each group to the mean of the control group.

After 1 week, do any of the additives have a significant effect on pro-collagen concentration produced by HDFs in dehydrated gels?

The pro-collagen concentration in cell culture medium from dehydrated hydrogels after 1 week was compared by a one-way ANOVA followed by a post-hoc Dunnett's

test. The results are presented in Table i.vii. Hyaluronic acid alone did not significantly increase the pro-collagen concentration while both Hyaluronic acid and Hyaluronic acid in combination with Ascorbic acid decreased pro-collagen concentration ($p<0.01$).

Table i.vii: Results of post-hoc analysis of a one-way ANOVA with a Dunnett's test. The concentration of pro-collagen produced by HDFs cultured for 1 week in dehydrated collagen gels. Gels were supplemented with HA, AA or both, were compared to dehydrated controls.

(I) Addition	(J) Addition	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
AA	Control	-1706.146	433.800	.002	-2780.269	-632.023
HA	Control	111.042	433.800	.987	-963.081	1185.165
AA+HA	Control	-1441.458	433.800	.007	-2515.581	-367.335

After 4 weeks, do any of the additives have a significant effect on pro-collagen concentration produced by HDFs in dehydrated gels?

After 4 weeks, supplementing gels with AA, HA and AA and HA in combination had a significant negative impact on pro-collagen concentration (Table i.viii) present in medium compared to control gels ($p<0.01$).

Table i.viii: Results of post-hoc analysis of a one-way ANOVA with a Dunnett's test. The concentration of pro-collagen produced by HDFs cultured for 4 weeks in dehydrated collagen gels. Gels were supplemented with HA, AA or both, were compared to dehydrated controls.

(I) Addition	(J) Addition	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
AA	Control	-25046.500*	7582.573	.008	-44060.219	-6032.781
HA	Control	-21026.929*	7848.709	.034	-40707.998	-1345.859
AA+HA	Control	-27964.167*	8190.115	.006	-48501.331	-7427.003