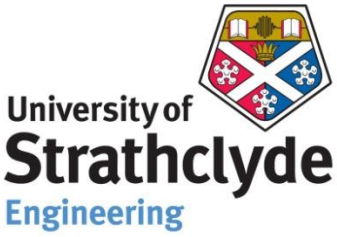


Manipulating the Mechanical Strength and Biological Stability of Collagen-based Scaffolds for Tissue Engineering

By

Grahame Busby

In partial fulfilment of the requirements for the degree of Doctor of Engineering

2013

University of Strathclyde

Department of Biomedical Engineering:

Doctoral Training Centre in Medical Devices

Declaration of Authenticity and Author's Rights

This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

Signed:

Date:

Acknowledgements

First and foremost, I would like to thank my lead supervisor Prof Helen Grant. I can't imagine how much more difficult this process would have been without her continued support, academic expertise, encouragement, enthusiasm, kindness... and patience. I would also like to thank my other supervisors; Dr Phil Riches, for expertly teaching me a great deal about an area of bioengineering I previously knew little about, and then continuing to support and encourage me as I came to terms with the magnitude of what I had taken on; and Prof Simon MacKay, for his knowledge and expertise, and for provoking thought and providing ideas whenever possible. I would also like to thank the EPSRC for funding my EngD research.

I would like to say a special thank you to Katie Henderson for looking after me in the lab, for providing me with the knowledge and support necessary to carry out my laboratory work, and for being a friend throughout. I would also like to thank Liz Goldie for her assistance (and persistence) with microscopy work, and her enthusiastic outlook. And I would like to thank everyone that worked with me throughout this process; whether in the cell lab, the tissue characterisation lab, or in the EngD room – you all made it an enjoyable and entertaining experience.

A huge thank you has to go to Georgina for putting up with me throughout this whole process. I know it wasn't easy, and it means a lot. I also want to thank my family (especially my parents) and my friends for continuing to support me, despite the fact I have become somewhat of a social recluse in more recent times.

Finally, and in no particular order, a few things that helped me out along the way: surfing, my guitars, snowboarding, good music, countless cups of tea, the internet (both a help and a hindrance), the Scottish climate (for making it easier to stay indoors for extended periods of time), skateboarding, single malt whisky, the beach, my bike, cold Guinness, and a few more cups of tea. Thanks. Stoked.

Collagen, the most abundant structural protein in the body, has become the most widely used matrix for tissue engineering. Implanted collagen undergoes a constant process of remodelling, and it is the balance of collagen synthesis and its gradual breakdown by the cells of the body that will determine its strength and effectiveness as a scaffold. It has been shown that collagen for implantation is degraded more quickly than cells are able to synthesise new collagen, resulting in a rapid reduction in matrix strength. The collagen is broken down by tissue/cell derived collagenases, which are members of the matrix metalloproteinase (MMP) family of enzymes.

Angiotensin converting enzyme (ACE) inhibitors have long been used successfully to treat hypertension. More recently, some ACE inhibitors have also been shown to inhibit certain MMPs. The purpose of this thesis was to investigate the potential of the ACE inhibitors captopril and enalapril to inhibit enzymatic collagen degradation, and ultimately slow down the rate at which collagen is degraded by cells of the body.

Both captopril and enalapril were shown to inhibit enzymatic collagen degradation in a dose-dependent manner, at concentrations that are non-toxic to cells in culture. To determine whether the ACE inhibitors were able to affect the mechanical properties of fibroblast-populated collagen lattices (FPCLs), a technique was developed for the characterisation of their time-dependent properties. The technique, which facilitates estimation of the stiffness and hydraulic permeability of hydrogel samples via confined compression and biphasic theory, may also be suitable for characterising other inherently weak hydrated tissues, and therefore may be of great value to anyone interested in doing so. Having demonstrated that the technique was sensitive to small variations in collagen content, it was used to compare the mechanical properties of FPCLs, where it was shown that FPCLs treated with captopril or enalapril were stiffer than control FPCLs after 6 days in culture. It seems likely that the retention of matrix stiffness is attributable to a reduction in the rate of substrate degradation by collagenolytic enzymes, which in turn can be attributed to their inhibition by the ACE inhibitors, though the mechanism of inhibition is still not fully understood. The potential to manipulate the strength and stability of collagen implants by treating them with ACE inhibitors has major implications throughout the fields of tissue engineering, regenerative medicine and cosmetic surgery.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF MATHEMATICAL NOMENCLATURE

c	Constant
E	Young's modulus
h	Sample height
H_A	Aggregate modulus
k	Hydraulic permeability
k_0	Zero-strain hydraulic permeability
M	Nonlinear permeability coefficient
t	Time
u	Displacement
v	Velocity
z	Axial coordinate
ΔP	Applied pressure difference
ΔZ	Thickness (of porous platen)
ε	Strain
λ	Stretch ratio
σ	Compressive stress
σ_{eq}	Equilibrium compressive stress
σ_{pk}	Peak compressive stress
υ	Poisson's ratio

LIST OF ABBREVIATIONS

3D	Three-Dimensional
ACE	Angiotensin Converting Enzyme
ANOVA	Analysis of Variance
AO	Acridine Orange
APMA	4-Aminophenylmercuricacetate
APS	Ammonium persulfate
BSA	Bovine Serum Albumin
CFDA	Carboxyfluorescein Diacetate
DMA	Dynamic Mechanical Analysis
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EPSRC	Engineering and Physical Sciences Research Council
EtBr	Ethidium Bromide
FDA	US Food and Drug Administration
FPCL	Fibroblast-Populated Collagen Lattice
GAG	Glycosaminoglycan
MMP	Matrix Metalloproteinase
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NA	Numerical Aperture
NR	Neutral Red
PBS	Phosphate Buffered Saline
PI	Propidium Iodide
PTFE	Polytetrafluoroethylene
PUV	Pulsed Ultra-Violet
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
TEMED	Tetramethylethylenediamine
TES	Tris(hydroxymethyl)methylamino)ethane-1-sulphonic acid
TIMP	Tissue Inhibitor of Metalloproteinases

Chapter 1

Introduction

1 Introduction

1.1 Tissue Engineering

The loss or failure of tissues and organs resulting from injury, disease, or genetic defect is a major human health problem. Transplantation of tissues or organs is the standard therapy for the treatment of many such conditions. The need for donor tissues or organs will continue to grow as modern medicine increases the human lifespan, leading to an aging population and ultimately a critical shortage of healthy donors. If suitable organs do become available for transplant, there is always a strong possibility that the organ will be rejected by the body, and even where organs are not rejected the need for the lifelong use of immunosuppressive medications can lead to a number of further complications in patients. Consequently, scientists have been looking to new fields for alternatives to tissue transplantation.

Tissue engineering, although a relatively new area for scientific research, has emerged as a promising approach to meet the ever increasing demand for organs and tissues (Chen *et al*, 2002; Olson *et al*, 2011). In the most basic sense, tissue engineering is concerned with the fabrication of living replacement parts for the body, and a commonly applied definition is that of Langer & Vacanti (1993), who defined it as "an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ".

1.2 Scaffolds for Tissue Engineering

While the term tissue engineering can be applied to artificially created support systems that use living cells to perform specific biological functions (e.g. bioartificial liver (Allen *et al*, 2001)), it more commonly refers to efforts to employ material scaffolds to serve as a synthetic extracellular matrix (ECM), sometimes containing cells or tissues grown outside the body, with the purpose of stimulating and organising cells into a three-dimensional (3D) architecture, directing the formation and growth of a desired tissue (Yang *et al*, 2001).

Tissue engineering strategies incorporating some form of synthetic ECM can often be divided into three basic strategies: conductive, inductive, and cell transplantation strategies (Silva & Mooney, 2004). Conductive strategies rely on synthetic matrices to promote population of a tissue defect site by host cells, resulting in the reformation of lost tissue. Inductive approaches concern the delivery of bioactive signalling molecules (e.g. growth factors) to specific cell populations in the tissues surrounding a defect site. Finally, cell transplantation strategies involve the delivery of specific cell populations, typically cultured *in vitro*, to the site at which new tissue development is required.

1.2.1 The Extracellular Matrix (ECM) as a Scaffold

The ECM is the structural material that underlies epithelia and surrounds the cells of connective tissue. It is manufactured by the resident cells of each tissue and organ, maintaining a state of dynamic equilibrium with its surrounding microenvironment (Badylak, 2007), and can be considered the ideal scaffold or biomaterial for tissue engineering. Not only do the molecules of the ECM provide a means by which cells within the matrix communicate with each other and the external environment (Brown & Dejana, 2003), but they also provide a physical supportive medium for nerves, lymphatics and blood vessels, and for the diffusion of nutrients within the matrix. The ECM is composed primarily of collagens, but also contains a variety of other molecules including elastic fibres, glycosaminoglycans (GAGs) and proteoglycans, and adhesive glycoproteins. It is the variations in amounts, combinations, spatial organisation, and immobilisation of these substances that determine the type of scaffold and its specific tissue function (Rosso *et al*, 2004).

Although biological scaffolds consisting of various forms of intact ECM have been used to aid and promote constructive remodelling of tissues (Abraham *et al*, 2000; Badylak, 2004; Robinson *et al*, 2005), it would be far more useful if a synthetic ECM could be fabricated *in vitro*. Unfortunately, the complex 3D organisation of the native ECM molecules is still not fully understood, and synthesis of a biologically similar biomaterial is not yet possible. However, in efforts to perform a variety of

biological functions, there have been many attempts to create scaffolds that are compatible with or mimic the ECM. In particular, hydrogels (biphasic matrices consisting of a solid fibrous network and interstitial fluid) have been a major focus for research, and some of the strategies for tissue engineering of hydrogels are discussed below.

1.2.2 Design Requirements of Hydrogels for Tissue Engineering

In order to promote new tissue formation and function appropriately there are a number of design criteria that hydrogels for tissue engineering must meet. The most critical of these is the requirement for biocompatibility of both the bulk and degraded forms of the hydrogel. The biocompatibility of a scaffold relates to its ability to perform a desired therapeutic function without eliciting any undesirable local or systemic responses that would detract from its intended purpose. Such effects could be of particular importance since an inflammatory response to a hydrogel can in turn influence the immune response towards transplanted cells and vice versa (Babensee *et al*, 1998; Říhová, 2000).

The hydrogels must provide an adhesion substrate for transplanted cells, with appropriate surface structures for cell attachment. In order to allow for high cell densities a large surface-area-to-volume ratio is desirable, and this in turn dictates the use of highly porous scaffolds. A highly porous matrix would also provide ports of entry for capillary transfer of nutrients into the scaffold, allowing for the engineered tissue to fully integrate with the host body's vascular supply and the subsequent convection and diffusion of metabolites within the tissue. Vascularisation is of the utmost importance, as the potential for nutrient transfer via diffusion is extremely limited, and it has been reported that cells become necrotic or at least metabolically inactive if located more than approximately 200 μm from a blood supply (Clark, 1995).

The mechanical properties of hydrogels are also of major importance. Scaffolds must be sufficiently strong to create and maintain a space for tissue development, and to

withstand *in vivo* forces. Furthermore, the hydrogel should provide the correct stress environment for the transplanted cells, as it has been shown that substrate stiffness affects factors such as cell adhesion and gene expression (Discher *et al*, 2005; Huang & Ingber, 1999), proliferation (Helary *et al*, 2010), and the direction of stem cell lineage specification (Engler *et al*, 2006). Controlled degradation of scaffolds is also desirable and, although scaffolds for long-term stability may be required not to degrade, the majority of hydrogels intended to promote repair or remodelling of tissues should be biodegradable. Furthermore, it is usually a requirement that the degradation rate of a scaffold is coordinated with the rate of neotissue development/synthesis (Lee *et al*, 2001c). Depending on the composition of the matrix, degradation is typically due to hydrolysis, dissolution, and/or the action of enzymes (Lee & Mooney, 2001).

1.2.3 Materials Selection for Hydrogels for Tissue Engineering

There are two main classes of biomaterial for fabricating exogenous ECMs: synthetic materials and naturally derived materials. Examples of synthetic materials used in the production of hydrogels for tissue engineering are poly(vinyl alcohol) (Grant *et al*, 2006), poly(acrylic acid) (Lee & Mooney, 2001), and poly(ethylene oxide) (Gunatillake & Adhikari, 2003). In addition, copolymers of poly(lactic-*co*-glycolic acid), polyglycolic acid, and poly(L-lactic acid) have been approved by the US Food and Drug Administration (FDA) for human clinical use (Gilding, 1981), and are widely used in tissue engineering (Kim & Mooney, 1998). Such synthetic materials are appealing to tissue engineers as they can be manufactured on a large scale, with readily controlled and reproducible chemistry and properties. Synthetic polymers with attributes such as specific molecular weights, degradable linkages and controlled crosslinking modes can be reproducibly produced, meaning that material properties such as crosslinking density, mechanical characteristics and degradation pathways can be predetermined (Drury & Mooney, 2003). In particular, synthetic hydrogels are usually degraded via chemical hydrolysis, and are therefore insensitive to enzymatic processes (Yang *et al*, 2001). However, a major disadvantage of synthetic polymers is their lack of cell-recognition signals and lack of functional

groups available for covalent modification. Many of these polymers also produce acidic degradation products, which could result in high local acid concentrations as they degrade (Gunatillake & Adhikari, 2003).

Naturally derived materials are frequently used for tissue engineering applications because they possess macro-molecular properties similar to those of the natural ECM (Drury & Mooney, 2003). In fact many natural hydrogel forming polymers, such as collagen, gelatin and hyaluronate, are components of the natural ECM. Such polymers are capable of specific cell interactions, and are completely biodegradable with no toxic by-products. However, these materials require to be isolated from human or animal tissue, and as such are generally not available in large quantities. Alternatively, hydrogel forming polymers can also be derived from non-animal sources, such as alginate or agarose, both of which are marine algal polysaccharides. These marine polymers have relatively low cost, low toxicity, and are biocompatible, though they suffer from problems with uncontrollable and unpredictable degradation processes (Lee & Mooney, 2001).

A major issue with all natural hydrogel forming polymers is the variation between batches that is often observed (Kim & Mooney, 1998). They also typically display a lack of mechanical strength and structural integrity, despite many investigations into methods to improve their mechanical properties (Drury & Mooney, 2003; Lee & Mooney, 2001). Although collagen is the most abundant natural component of the ECM, and therefore would seem ideal for tissue engineering purposes, it too suffers from poor mechanical strength, and is degraded more quickly by the cells of the body than they are able to synthesise new ECM (Saddiq *et al*, 2009). This study will therefore focus on the use of collagen for tissue engineering, and in particular on methods to manipulate and control its mechanical properties via inhibition of degradative enzymes.

1.3 Collagen

1.3.1 Collagen Biochemistry and Structure

Collagen is the most abundant structural protein in the mammalian body, accounting for up to 30% of total body proteins (Harkness, 1961). It is primarily a component of tissues that provide a mechanical function, imparting physical support by occupying the intercellular space, and accounts for around 70% of the non-aqueous material present in dermis of skin and tendon (Lee *et al*, 2001a). Collagen is the main constituent of the natural ECM, allowing for cell attachment in addition to imparting the strength that enables the shape of tissues to be defined and maintained (Kadler *et al*, 1996).

Collagens are a family of proteins, of which there are at least 28 different types that occur in vertebrates (Kadler *et al*, 2007). Each type exhibits differences in structure, splice variants, the presence of non-helical domains, tissue distribution and function (*Table 1.1*) (for a detailed review see Gelse *et al* (2003)). Despite these differences, all the variants share a similar triple helical structure formed from three intertwined α-chains. The three α-chains are held together by interchain hydrogen bonds, and their winding together gives collagen its strength. Each polypeptide chain has a repeating Gly-X-Y triplet, where the X and Y positions are frequently proline and 4-hydroxyproline respectively, and glycyl residues occupy every third position *(Fig. 1.1(a))* (Kadler *et al*, 2007). The stability of collagen is at least partly attributable to glycine having the smallest side group, which, in combination with its repetition, allows for close packing of the α-chains, leaving little space for residues in the core (Friess, 1998). The end of the chains contain short, non-helical regions which allow for crosslinking, further enhancing the strength collagen derives from its triple helical structure (Kühn, 1985). The majority of collagens are homotrimers (i.e. containing three identical α-chains), though they can also be heterotrimers, such as collagen I, which contains two identical α-chains and one that differs, $[\alpha1(I)]_2$ α2(I) (Kadler *et al*, 2007).

Type	Family	Tissue Distribution
I, II, III, V, XI	Fibril-forming collagens	Bone, dermis, tendon, ligaments, cornea, cartilage, vitreous body, nucleus pulposus, skin, vessel wall, reticular fibres, lung, foetal membranes
IV	Basement membrane collagens	Basement membranes
VI	Microfibrillar collagens	Dermis, cartilage, placenta, lungs, vessel wall, intervertebral disc
VII	Anchoring fibrils	Skin, dermal-epidermal junctions, oral mucosa, cervix
VIII, X	Hexagonal network-forming collagens	Endothelial cells, Descemet's membrane, hypertrophic cartilage
IX, XII, XIV, XIX, XX, XXI	Fibril Associated Collagens with Interrupted Triple Helices (FACIT)	cartilage, vitreous humor, cornea, perichondrium, ligaments, tendon, dermis, vessel wall, placenta, lungs, liver, embryonic skin,
XIII, XVII	Transmembrane collagens	epidermis, hair follicle, endomysium, intestine, chondrocytes, lungs, liver, dermal– epidermal junctions
XV, XVI, XVIII	Multiplexins	fibroblasts, smooth muscle cells, kidney, pancreas, amnion, keratinocytes, lungs, liver

Table 1.1: Collagen types, families, and tissue distribution (Gelse *et al*, 2003)

Despite the variations between groups within the collagen family (see *Table 1.1*), the most abundant group are the fibril-forming collagens (types I, II, III, V and XI), which have large sections of homologous sequences independent of species (Lee *et al*, 2001a). The collagen fibrils, which individually are around 300 nm in length, aggregate into larger collagen fibres, which then associate further with one another to create even larger collagen fibres *(Fig. 1.1)* (Friess, 1998; Kadler *et al*, 1996). The process of fibril formation is not fully understood, though it appears to be dependent on factors including the cleavage of collagen propeptide sequences and the formation of covalent cross links (Brown & Phillips, 2007). It is this group of collagens that appear to offer the most potential for use in tissue engineering, and in particular collagen type I, on which the majority of materials for biomedical applications are based due to its abundance in higher order animals (Friess, 1998).

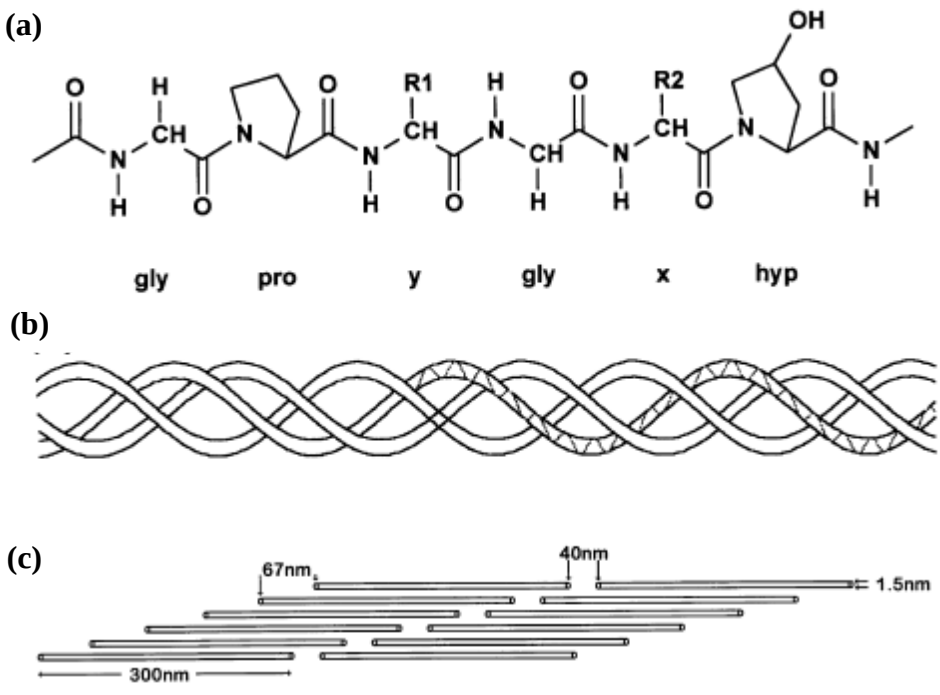


Figure 1.1. Structure of collagen type I. (a) Primary amino acid sequence, (b) tertiary triple-helix structure of a collagen fibril, (c) collagen fibrils staggered together to create collagen fibres, 67 nm apart and crosslinked. Modified from Friess (1998).

1.3.2 Collagen as a Biomaterial

1.3.2.1 Advantages/Disadvantages of Collagen as a Biomaterial

The abundance of collagen in the mammalian ECM is an important factor when considering its suitability as a biomaterial. Collagen is relatively easy to isolate from tissues, and the fact that it is so prevalent in the tissues of living organisms means that it is widely available, and therefore cost effective for experimental use. It is also extremely biocompatible, non-toxic, and only very weakly (if at all) antigenic (Lee *et al*, 2001a). It can also be modified with relative ease by utilisation of its functional groups (Lee *et al*, 2001a).

One of the most useful properties of collagen is its biodegradability, which is mainly due to denaturation of the protein by collagenase enzymes (discussed in **section 1.5.2**). In tissue engineered applications this process is necessary to ensure that new *native* ECM can be constructed in place of the engineered scaffold. However, this degradation must be controlled and regulated if the rate of neotissue growth is to be matched to the rate of enzymatic degradation, and a common problem with collagen-based scaffolds for tissue regeneration is that they are degraded more quickly than cells are able to synthesise new ECM materials, resulting in a loss of overall tissue strength and stiffness (Saddiq *et al*, 2009). It is hypothesised in the present study that this imbalance of protein synthesis and degradation could be a consequence of the over activity of degradative enzymes, and that the stiffness of collagen-based scaffolds could potentially be controlled by inhibition of these enzymes.

1.3.2.2 Collagen-based Scaffolds for Tissue Engineering

Collagen can be produced in many different forms such as films, shields, pellets and nanoparticles (Lee *et al*, 2001a). However, none of these forms are particularly suited to producing scaffolds that are intended to promote cellularisation. In contrast, there are some approaches that much better lend themselves to producing collagenous cell-based scaffolds. *Fibrous scaffolds*, for example, rely on the extraction of insoluble collagen fibres from native ECM, which are then reassembled into scaffolds with the

desired properties, such as nerve repair conduits made from commercially available type I collagen filaments (Koken Co., Ltd., Japan) (Yoshii & Oka, 2001; Yoshii *et al*, 2003).

Collagen *sponges/foams* have also shown considerable promise as tissue engineered constructs and are already well established, having been used clinically in acellular forms as wound dressings for many years (Sheridan *et al*, 1994; Yannas & Burke, 1980). Collagen sponges can absorb large volumes of tissue exudates, adhere well to the wet wound bed whilst preserving moisture, and also act as a shield against mechanical harm and infection (Yannas, 1990). They can be used for the controlled release of drugs or growth factors into tissues, in addition to supporting cell ingrowth, and therefore actively promote the healing process (Friess, 1998; Lee *et al*, 2001a). They may also be useful for the directed delivery of cells into tissues, although a major issue with such an approach would be their dissimilarity to native ECM tissues, particularly in terms of their mechanical characteristics. This unfamiliar architecture means that cells within the scaffolds are unlikely to perceive the kind of mechanical cues necessary to conduce the alignment required in many tissues (Brown & Phillips, 2007), and so their most useful applications are in tissues where alignment is not necessarily critical for function, e.g. skin replacement.

Probably the most widely used forms of collagen for tissue engineering strategies are collagen *hydrogels*. Collagen hydrogels have a long history as potential scaffolds for tissue repair and modelling (Bell *et al*, 1979; Elsdale & Bard, 1972), and were chosen for investigation in the present study. As such, they are discussed in detail in **section 1.4**.

1.3.2.3 Collagen-based Scaffolds as Delivery Systems

Although the field of tissue engineering is most commonly concerned with strategies for tissue remodelling and replacement, there are other applications for which the advantages of biological scaffolds can be exploited. In fact, collagen-based scaffolds have more commonly been used medically as drug and gene delivery systems than as

matrices for tissue engineering (Friess, 1998; Lee *et al*, 2001a). Drugs can be loaded into collagen scaffolds by covalent or hydrogen bonding, or by entrapment, and many drug delivery systems using a variety of collagen-based scaffolds have already been developed and utilised (for a detailed review see Friess (1998)).

1.4 Collagen Hydrogels for Tissue Engineering

Collagen hydrogels have a long and extensive history of being used in studies of cell culture, tissue modelling and tissue engineering (Bell *et al*, 1979; Elsdale & Bard, 1972; Grillo & Gross, 1962; Grinnell, 1994). Although they can be used for the sustained delivery of soluble factors, the openness of the network requires that secondary mechanisms often be employed to retain active agents, adding to the complexity of the system, and so their main potential uses are as substrates for the growth and delivery of cells, or as space filling agents (reviewed by Wallace & Rosenblatt (2003)). They can be easily manufactured because purified fibrillar collagen (typically but not necessarily type I) in acidic solution forms a stable gel when adjusted to physiological pH and temperature (Elsdale & Bard, 1972; Gross & Kirk, 1958). Collagen hydrogels set quickly at physiological pH (< 30 min), meaning that they can be easily moulded into shape. They can also be seeded with cells while still in liquid form, ensuring that cells are distributed throughout the substrate (and not only on the surface) when the gel is formed. Another major advantage is the potential for delivery into warm-blooded mammals, since they can be easily injected at room temperature but will become rigid after a short time in living tissue, ensuring that the gel resists displacement from the site of application.

The hydrogels themselves are biphasic in their construction, consisting of an interconnected network of collagen fibres of indeterminate length and varying thickness held together by hydrophobic and electrostatic bonds, and filled with a large excess of interstitial fluid (typically > 99%). Unfortunately, the very high water content means that they are inherently very compliant and weak structurally, and this lack of mechanical stiffness and strength, in addition to their lack of a specifically

oriented architecture, means that they are unsuitable for many tissue engineering applications.

1.4.1 Improving Mechanical Characteristics of Collagen Hydrogels

One method commonly employed to create collagen hydrogel scaffolds of increased stiffness is via cellular contraction. Cells (typically fibroblasts) cultured with collagen hydrogels spread and elongate as they migrate through the matrix, contracting the hydrogel as they do so, resulting in a smaller and more densely packed scaffold (Grinnell, 2003). Contracted collagen hydrogels seeded with fibroblasts have been studied as wound models (Grinnell, 1994), and are often considered to be appropriate dermal equivalents (Bell *et al*, 1979). In fact Apligraf® (Organogenesis Inc., USA), a commercially available dermal equivalent consisting of a fibroblast contracted collagen hydrogel and an upper epidermal layer formed from differentiated human keratinocytes, has achieved considerable clinical success in the treatment of leg ulcers and other skin wounds (Allie *et al*, 2004; Falanga, 1998; Kirsner, 1998). The mechanistic actions of cells can also be used to confer alignment to scaffolds, since cells and collagen fibrils will align themselves with the direction of maximum principal strain (Eastwood *et al*, 1998; Mudera *et al*, 2000). Consequently, aligned cellular constructs can be formed by securing the hydrogels only at each end, i.e. under uniaxial tension. While these cell-based techniques have shown considerable promise, they are not always suitable. Although the contraction results in stiffer gels, it also reduces the permeability of the scaffold, can initiate apoptosis, and prohibits control of the resulting size and shape of the construct (Gentleman *et al*, 2004). Cell-contracted scaffolds take several days to prepare, and require that scaffolds are initially much larger than the treatment area (Grinnell, 1994). It is also not always suitable to use cell-seeded scaffolds, particularly where they are primarily intended as space-filling agents. However, several other methods for strengthening collagen matrices have also been developed, and some of these are discussed herein.

One approach to strengthening collagen matrices is to prepare them as composites with other stiffer components, such as embedded collagen fibres (Gentleman *et al*, 2003; Gentleman *et al*, 2006). However, the most common method of increasing the mechanical and biological stability of collagen hydrogels is through increased crosslinking within the substrate, which can be achieved in a number of ways. The hydrogels can be crosslinked by physical treatments, e.g. ultraviolet irradiation or dehydrothermal treatment, or by treatment with chemical crosslinkers like glutaraldehyde or carbodiimide (Lee *et al*, 2001b; Park *et al*, 2002). The addition of glycosaminoglycans (GAGs) has proved particularly successful, and their usage would appear appropriate because GAGs are normal constituents of the ECM, and thus scaffolds containing GAGs would more closely mimic the natural matrix. Increases in the mechanical stability of collagen sponges crosslinked with the GAG chondriotin-6-sulphate ($Ch6SO_4$) were demonstrated in 1990 by Matsuda *et al* (1990). Saddiq *et al* (2009) then demonstrated that $Ch6SO_4$ strengthens acellular collagen hydrogels considerably, although they also reported that the addition of chemical crosslinkers did nothing to increase the mechanical properties of hydrogels that had been cultured with human fibroblasts for 6 days. In fact, it has been suggested that it is the presence and activity of cells and secreted enzymes, rather than the chemical composition of collagen-based matrices, that determine a scaffold's mechanical properties (Friess, 1998; Saddiq *et al*, 2009). Although additional crosslinks can clearly improve the mechanical stability of collagen-based structures, they do not appear able to ensure an adequate balance between enzymatic degradation and synthesis of new ECM material.

More recently a technique has emerged that allows the manufacture of fibrillar collagen scaffolds with mechanical properties approaching those of native collagen tissues. It is known as Plastic Compression, and involves the rapid expulsion of the majority of the hydrogel fluid under unconfined compression (Brown *et al*, 2005). The result is a thin sheet of fibrillar collagen with functional mechanical properties, which can then be layered and/or rolled to create a variety of structures (Nazhat *et al*, 2006). They are also highly cell-supportive, and cells can survive the compression process meaning that they can be seeded at the gel stage (Brown *et al*, 2005). Furthermore, possibly the main advantage of this technique is the speed at which

mechanically strong cell-populated scaffolds can be assembled. However, the technique is not without limitations; namely that collagen that has been plastic compressed is no longer injectable and so could not be administered in this way.

Despite these advances in techniques to increase matrix stiffness, there still appears to be a requirement for methods to slow down the rate at which collagen hydrogels are degraded by cell-secreted enzymes, and especially if the desired modifications can be implemented prior to the gelling stage.

1.4.2 Mechanical Characterisation of Hydrogel Scaffolds

Ideally, the rate of degradation of a scaffold by the cells of the body should be matched to the rate of new tissue formation. It is therefore desirable to be able to control and manipulate this rate of matrix degradation. However, in order to ascertain whether a particular treatment is having an effect on the mechanical properties of a scaffold it is also necessary to find ways in which to characterise these properties, which can be particularly difficult due to the often very weak and poroelastic nature of hydrogels. Several techniques to determine the mechanical properties of hydrogels have been proposed, and some of the techniques most appropriate to the mechanical characterisation of specifically collagen hydrogels are discussed herein, along with their relative merits. Some of the factors that contribute to variation in the practical measurement of collagen hydrogel mechanical properties, and how these factors can be controlled, are then discussed.

1.4.2.1 Techniques for Mechanical Characterisation of Hydrogel Scaffolds

Extensometry is often employed in hydrogel characterisation, where uniaxial tensile tests are carried out using a conventional testing machine equipped with two sample grips and an appropriate load cell (Huang *et al*, 1993; Osborne *et al*, 1998; Saddiq *et al*, 2009). The scaffold to be tested is typically deformed at a constant strain rate, and the force required to maintain that strain rate is recorded. These measurements can

then be used to prepare a stress versus strain chart, from which mechanical parameters such as the Young's modulus, ultimate tensile strength and yield strength can be derived. Dumbbell-shaped samples are often employed in these tensile tests because when strips of uniform width are tested it is likely that the sample will fracture at the point of contact with the grips (where stress concentrations would be high), and so the value obtained for the stress at failure will not correlate with the ultimate tensile strength (Feng *et al*, 2003b; Saddiq *et al*, 2009). In contrast, dumbbell-shaped samples are more likely to fail in the mid-section. Unfortunately a major issue with this type of testing is that samples are tested to destruction, so repeated tests on the same sample are not possible.

The mechanical properties of collagen gels have also been tested using a Stevens Texture Analyser, a device commonly used to test mechanical properties of tissues as foodstuffs (Muyonga *et al*, 2004). A probe of fixed diameter is moved at a constant velocity into the gel, and the load required to penetrate the gel is recorded (Coviello *et al*, 2003). Again, this method is destructive, and will only provide data on the properties of a small area of the sample.

Other common testing methods are those concerned with the compression of samples. In the most basic form a porous indenter is used to apply a force vertically to an area of the hydrogel and the Young's modulus is determined by comparison of the applied force and the observed deformation of the gel. In unconfined compression the force required to uniaxially compress the hydrogel between two impermeable plates is measured, in addition to the amount of axial deformation (Koob & Hernandez, 2003; Stammen *et al*, 2001). An appropriate stress versus strain graph can then be derived, and the compressive strength and compressive modulus determined. A major limitation of this type of testing is that in order to determine the compressive modulus, it must be assumed that there is zero friction at the boundary. Although friction is extremely difficult to remove, it can be neglected if the samples are axially long compared to their radial length. Unfortunately such dimensions would be difficult to achieve for inherently weak hydrogels. In contrast, during confined compression testing the hydrogel is confined to a chamber, preventing lateral deformation of the sample as it is compressed by a porous platen (Behravesh

et al, 2002; Gu *et al*, 2003). Comparison of these compressive testing techniques has led researchers to conclude that the equilibrium response of certain biomaterials cannot be universally characterised using isotropic modelling, and that values of elastic parameters are dependent on the measurement technique used (Korhonen *et al*, 2002).

In bulge (blister) testing a uniform fluid pressure is applied to a thin film window of the substrate in order to deform the membrane, and a compliance curve is then produced by measuring the displacement as a function of the applied pressure (Ranta-Eskola, 1979). Gripping problems common to extensometry can be eliminated, and in contrast to the testing methods already mentioned the stress of the deformed sample is bi-axial and axisymmetric. However, nuisances such as dissolved air or moisture when using fluid as a pressurised medium, and the potential for leakage, may cause error in compliance measurements leading to significant inaccuracies in characterisation of membranes (Briscoe & Panesar, 1991).

Rheometers, traditional equipment for measuring fluid characteristics, have been used to calculate the viscoelastic properties of hydrogels by examining the relationships between deformations and stresses and their derivatives (Jiang *et al*, 1999; Meyvis *et al*, 1999). Rheological measurements are typically performed in shear (lateral deformations), and while they have been used to characterise collagen hydrogels (Knapp *et al*, 1997), such an approach could be considered inappropriate for tissues that are more often exposed to compressive or tensile forces *in vivo*. Similarly, Dynamic Mechanical Analysis (DMA) can also be performed to measure the viscoelastic behaviour of materials (Brandl *et al*, 2007). DMA requires the application of a sinusoidal shear load on the sample, often via a porous indenter as described previously (Korhonen *et al*, 2002). A stress transducer measures the applied shear stress, while a strain transducer measures the strain induced in the sample. These variables are then used to obtain the complex shear modulus, which is related to the Young's modulus and can therefore be used to determine the stiffness of the material (Brandl *et al*, 2007).

More recently a novel experimental technique enabling the characterisation of the viscoelastic properties of polymer membranes has emerged. An instrument was

created consisting of two parts; a sample holder with a spherical indenter, and a system for image acquisition (Ahearne *et al*, 2005; Ahearne *et al*, 2008). The membrane for testing is circumferentially clamped within the sample holder and is deformed centrally using a ball of known weight, while the corresponding displacement at the centre is measured by a long-working-distance microscope with a computer linked charge-coupled device camera. Some advantages to using this method are that the deformed samples are bi-axial and axisymmetric, the method is applicable to permeable and semi-permeable membranes, and the force and displacement resolution can be extremely accurate. Moreover, the method is non-destructive and can be performed for specimens fully immersed in solutions and at elevated temperatures without risk of damaging the instrument. However, the technique also appears extremely complex and time consuming, and is unlikely to facilitate throughput of a high sample numbers.

Other non-destructive methods of tissue characterisation are being explored, such as atomic force microscopy and magnetic resonance elastography (Brandl *et al*, 2007). However, such techniques are still far from being routine in tissue engineering and further research into the application of these methods for tissue characterisation is required.

1.4.2.2 Intrinsic Variation in the Mechanical Characterisation of Collagen Hydrogels

Pure collagen is very expensive, and consequently it is often produced ‘in house’ by those who require the material for study (Lee & Mooney, 2001). Unfortunately, there are often variations in collagen content between batches, and it is thought that these variations could lead to differences in the mechanical properties of collagen gels, particularly as it has been suggested that the apparent stiffness of the matrix is dependent on the collagen concentration (Brandl *et al*, 2007; Krishnan *et al*, 2004),. Furthermore, prepared collagen may contain impurities, and these can also vary between batches. It should therefore be ensured that gels to be compared are all produced from the same batch of collagen. It has also been suggested that the

apparent matrix stiffness of hydrogels varies when different types of endothelial cells are used to contract the matrix (Brandl *et al*, 2007), and thus it is important that where cells are used the same cell line is consistently used throughout testing, and that the cells are continuously observed to ensure they are not differentiating and adopting new characteristics.

The effects of temperature should be taken into consideration, as many hydrogels are likely to possess mechanical properties that vary with changes in temperature. It is therefore essential to ensure that samples for testing are thermally regulated throughout. Swelling conditions will also have an effect on matrix properties, as a higher degree of swelling is said to result in a reduction in matrix stiffness (Anseth *et al*, 1996). Although swelling conditions are often predetermined by the application requirements, it is important that measurement conditions are as close to *in situ* conditions as possible. Therefore, samples should be cut in their equilibrium swollen state and sample dimensions measured in this swollen state. For certain methodologies, such as tensile testing, the sample should also be fully immersed in a bath of solution in which the gel is at equilibrium. It has also been suggested that for testing methods that do not allow for samples to be immersed in solution during testing water loss can be minimised by coating the gel with petroleum gel or silicone vacuum grease (Anseth *et al*, 1996), although this approach would not be appropriate for certain tests of time-dependent properties (e.g. confined compression) where the movement of fluid out of the material is fundamental to its characterisation.

Finally, gelling conditions can also have a considerable effect on mechanical properties, and it is essential that all gels are fabricated at the same temperature and pH if structural characteristics are to be compared (Drury & Mooney, 2003).

1.5 Matrix Metalloproteinases (MMPs) and their Inhibitors

The MMPs are a family of more than twenty membrane-bound and secreted zinc-endopeptidases that require Ca^{2+} for activity (Visse & Nagase, 2003). Collectively MMPs have the ability to degrade all the components of the ECM, such as fibrillar

and non-fibrillar collagens, elastin, laminin, fibronectin and basement membrane glycoproteins. As a result, the activity or inhibition of these enzymes will have an effect on the structural composition, and therefore on the mechanical properties, of tissues. Indeed, it has been suggested that the loss of mechanical stiffness observed in anchored cell-seeded collagen matrices is at least partly attributable to the actions of these enzymes (Saddiq *et al*, 2009).

1.5.1 Function and Structure of MMPs

A typical MMP consists of four distinct regions: a propeptide, a catalytic metalloproteinase domain, a linker (or 'hinge region') and a hemopexin domain. The catalytic domains of all MMPs are similar in structure, consisting of a 5-stranded β-pleated sheet, three α-helices and a connective loop in addition to two zinc chelating sites (one catalytic and one structural) and up to three stabilising calcium ions *(Fig. 1.2)* (Nagase *et al*, 2006). Substrate binding is dictated mostly by the structure of the substrate binding sites, which are hydrophobic pockets of varying depth. The most common of these is the "S1′ pocket", located to the right of the active-site zinc, and the size of this site in particular is known to be one of the major factors determining substrate specificity (Visse & Nagase, 2003).

MMPs are synthesised as inactive zymogens (proMMPs) that must first be activated by proteinase cleavage. They are kept inactive by an interaction between a zinc ion bound to the catalytic domain and a cysteine-sulphydryl group (the "cysteine switch") in the propeptide domain, and their activation requires proteolytic removal of the propeptide domain (Sternlicht & Werb, 2001). Their activation is therefore an important regulatory step in MMP activity. The proMMPs possess a "bait" region that allows activation by tissue and plasma proteinases or opportunistic bacterial proteinases (Nagase & Woessner, 1999). MMPs can also be activated *in vitro* by chemical agents (e.g. 4-aminophenylmercuricacetate (APMA)), by lowering pH and by heat treatment (Visse & Nagase, 2003).

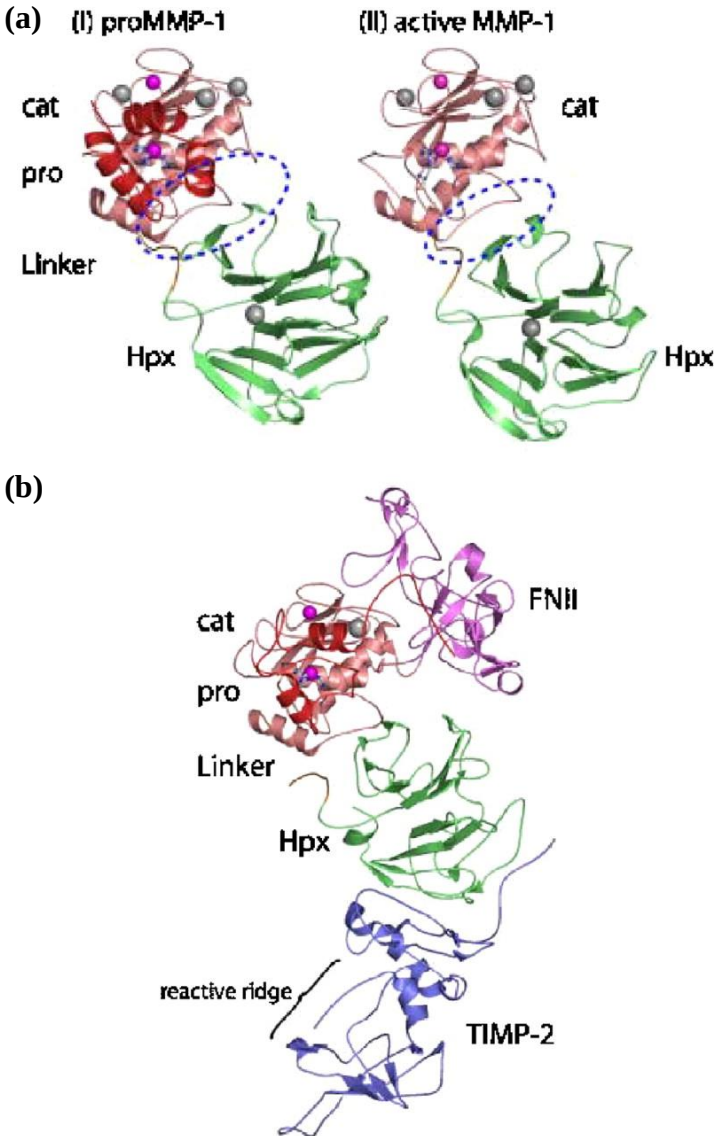


Figure 1.2. (a) Ribbon diagram of human proMMP-1 and active pig MMP-1. The pro-domain is shown in red, catalytic domain in pink, linker region in yellow, hemopexin domain in green, zinc ions in purple and calcium ions in grey. The dotted circle indicates the region where the catalytic and hemopexin domains intereact. Note that proMMP-1 has a larger area of contact sites than MMP-1. This results in the active form having an ‘‘open’’ configuration compared to the ‘‘closed’’ configuration of proMMP-1. It is predicted that this region is where triple helical collagens binds. (b) Ribbon structure of the complex of proMMP-1 and TIMP-2. Domains of proMMP- 2 are shown as in (a) and the finbronectin type II motif is in purple. TIMP-2 is shown in blue. From Nagase *et al* (2006).

1.5.2 Collagen Cleaving MMPs (Collagenases)

Three members of the family, MMP-1, MMP-8 and MMP-13, have been specifically identified as encoding mammalian collagenases i.e. enzymes whose main function is the degradation of collagen *in vivo*, although several other MMPs have also been ascribed with collagenolytic activities (*Table 1.2*). The key feature of the collagenases is their ability to cleave native collagens to produce approximately three-quarter and one-quarter fragments. At physiological temperatures these products spontaneously denature further, becoming susceptible to cleavage by a range of other less specific MMPs (Johnson *et al*, 1987). Therefore, by affecting only one specific cleavage, the collagenases initiate the degradation of the entire macromolecule. The upshot of this is that collagen degradation is critically controlled by collagenase activity, and hence inhibition of this activity should allow for modulation of collagen turnover.

MMP Family	MMP	Descriptive Name	Principal Collagen Substrate
Collagenases	MMP-1	Interstitial collagenase	Collagen types I, II, III (fibrillar collagens)
	MMP-8	Neutrophil collagenase	
	MMP-13	Collagenase-3	
	MMP-18	Xenopus collagenase	
Gelatinases	MMP-2	Gelatinase A	Types IV, V (nonfibrillar collagens)
	MMP-9	Gelatinase B	
Stromelysins	MMP-3	Stromelysin-1	Nonfibrillar collagens
	MMP-10	Stromelysin-2	
	MMP-7	Matrilysin	
Elastase	MMP-12	Metalloelastase	Nonfibrillar collagens

Table 1.2: Collagen cleaving matrix metalloproteinases (Nelson *et al*, 2000)

1.5.3 MMPs in Disease

Degradation of the ECM is precisely regulated under normal physiological conditions, and is an important feature of tissue development, morphogenesis, repair and remodelling. However, the dysregulation of MMP activity is associated with the pathological progression of several diseases, leading to increased interest in the subject in recent years. Although it has long been postulated that MMPs play a critical role in the progression of joint and bone destruction in diseases such as rheumatoid arthritis and osteoarthritis (Wojtowicz-Praga *et al*, 1997), it has more recently become apparent that uncontrolled ECM remodelling of the vasculature and myocardium are key features of cardiovascular disorders (e.g. atherosclerosis, left ventricular hypertrophy etc.) (Nagase *et al*, 2006). Furthermore, MMPs have been

associated with the progression of certain human malignancies, and are said to play a critical role in cancer-cell growth, invasion, migration, metastasis and angiogenesis (for a review of the functions of MMPs in cancer see Egeblad & Werb (2002)). In particular, MMP-2 and MMP-9 have for some time been implicated in the progression of certain cancers (Brown *et al*, 1993; Liabakk *et al*, 1996; Zucker *et al*, 1993), and as such have been a focus of much of the research into synthetic MMP inhibitors (Overall & López-Otín, 2002).

1.5.4 MMP Inhibitors

MMP activity *in vivo* is regulated by two main classes of endogenous inhibitors: α_2-macroglobulin and the tissue inhibitors of metalloproteinases (TIMPs). α_2-macroglobulin is a large (~750 kDa) and non-specific inhibitor of MMPs that is produced in the liver and acts by entrapping the enzyme, blocking its access to protein substrates (Nagase *et al*, 2006). Although α_2-macroglobulin is effective as an intravascular MMP inhibitor, its large size may decrease its potential for tissue penetration and limit its effectiveness elsewhere. Alternatively, TIMPs are a family of four specific MMP inhibitors (TIMP-1, TIMP-2, TIMP-3, and TIMP-4) that bind to the active sites of MMPs with no identifiable specificity (Bode *et al*, 1999a). The general shape of the TIMP molecules can be considered like a wedge, which slots into the entire length of the active-site cleft of MMPs in a manner similar to that of the substrate *(Fig. 1.2(b))* (Bode *et al*, 1999b). Although the therapeutic application of TIMPs has been investigated, it has been suggested that they are most likely not suitable for pharmacologic applications due to their short half-life *in vivo* (Wojtowicz-Praga *et al*, 1997).

A number of synthetic MMP inhibitors have also been studied, particularly in response to the common over-expression of MMP-2 and MMP-9 in highly aggressive human tumours. Much of the research has focussed on hydroxamate-based compounds that have structures that mimic collagen (Wojtowicz-Praga *et al*, 1997). Batimastat and marimastat are two extremely potent broad spectrum MMP inhibitors that, despite promising early animal studies, did not perform well in

clinical trials for cancer therapy (Coussens *et al*, 2002). Furthermore, they both exhibit very poor solubility (Wojtowicz-Praga *et al*, 1997), which could severely limit their efficacy in other applications such as the control of tissue degradation. Several other synthetic inhibitors have since been designed as anti-cancer agents, but poor performance in trials seems to be a common theme to any that make it to the clinical stage (for a detailed review see Overall & López-Otín (2002)). It is thought that the poor performance of synthetic MMP inhibitors in clinical trials could be due to their late application and that they may play a less important role once metastases have been generated, since it has been established that the role of MMPs is more prevalent in the early stages of cancer progression (Overall & López-Otín, 2002). It is also true that many synthetic MMP inhibitors are non-specific, and therefore may also target MMPs that prevent tumour progression (Coussens *et al*, 2002). There have also been a few studies into the clinical potential of MMP inhibitors to treat rheumatic disorders, although with little apparent success and no evidence of more recent work in the area (Cawston & Rowan, 1998; Elliott & Cawston, 2001). One particular drug that could have been of interest in the present study is cipemastat, an MMP-1 selective inhibitor developed by Roche Pharmaceuticals (Basel, Switzerland) that is unfortunately no longer available, presumably due to poor performance in clinical trials (Shaw *et al*, 2000). Nonetheless, the main focus of MMP inhibitor research to date has been in the fields of cardiovascular disorders and cancer therapy, and their potential therapeutic application in other areas, such as tissue engineering and wound repair, should not yet be ruled out.

1.6 Angiotensin Converting Enzyme (ACE) Inhibitors

ACE inhibitors are a large family of drugs that have been successfully used for many years in the treatment of hypertension (Cushman & Ondetti, 1999; Ferreira, 2000). There are numerous therapeutically acceptable ACE inhibitors that when taken orally all share the same biological effects of increasing venous capacity and cardiac output, lowering arterial resistance, and increasing sodium excretion (Brown & Vaughan, 1998). However, more recently, greater attention has been paid to the additional ability of some ACE inhibitors to inhibit MMPs. It was discovered some

time ago that captopril, the first and smallest ACE inhibitor to be developed, also had an inhibitory effect on MMP-2 and MMP-9 activities (Sorbi *et al*, 1993). Although these early studies were primarily concerned with the role of MMPs in nephropathy, the potential for ACE inhibitors to inhibit MMPs has since been studied with respect to a number of different physiological conditions. Due to their ability to increase blood flow ACE inhibitors are already a common treatment for recovery from heart failure, but now considerable attention is also being paid to their MMP inhibitory properties, particularly with respect to left ventricular remodelling following myocardial infarction (Brower *et al*, 2007; Guo *et al*, 2008; Jin *et al*, 2007; Reinhardt *et al*, 2002). They have also been investigated as anticancer agents, demonstrating an ability to reduce tumour growth and metastasis in animal models (Prontera *et al*, 1999; Williams *et al*, 2005).

Modelling studies have demonstrated similarities in the molecular structures of ACE inhibitors and the active sites of MMPs (Jin *et al*, 2007; Yamamoto *et al*, 2007b; Yamamoto *et al*, 2008). It is thought that the most plausible mode of action for MMP inhibition by ACE inhibitors is via the chelation of Zn^{2+} at the active sites, as it has been shown that the presence of excess Zn^{2+} can reverse the inhibitory effect to selected MMPs (Sorbi *et al*, 1993). It is also postulated that functional groups within the ACE inhibitors interact hydrophobically with amino acids in the catalytic domain of MMPs, blocking substrate access (Yamamoto *et al*, 2007a). These hydrophobic interactions are thought to be necessary to stabilise structurally larger ACE inhibitors (e.g. lisinopril, enalapril) while they occupy the active site (or sites) of MMPs (Yamamoto *et al*, 2008). However, it has also been suggested that the hydrophobic interactions are not as critical for the MMP inhibitory action of captopril, which lacks many of the large functional groups present in other ACE inhibitors, leading to more potent MMP inhibition (Yamamoto *et al*, 2008).

1.6.1 ACE Inhibitors Used in the Present Study

The ACE inhibitors captopril and enalapril were chosen in the present study for the purpose of evaluating the potential of ACE inhibitors to inhibit collagenase.

Captopril was the first ACE inhibitor to be developed, is the smallest structurally, and is one of the few that contains a sulfyhdryl group *(Fig. 1.3(a))*. Captopril was chosen as it is readily available for research purposes and has already been shown to be a potent inhibitor of MMP-2 and MMP-9 (Sorbi *et al*, 1993; Yamamoto *et al*, 2008), and so was hypothesised to be a likely inhibitor of interstitial collagenase (MMP-1) also. Enalapril is a much larger dicarboxylate-containing agent *(Fig. 1.3(b))*, and is a prodrug that must be converted to its active form, enalaprilat, by hepatic de-esterification (Baba *et al*, 1990). Enalapril was chosen as it is also readily available, and is very similar in structure to another ACE inhibitor, lisinopril, which has also been shown to inhibit certain MMPs (Sorbi *et al*, 1993; Yamamoto *et al*, 2007b).

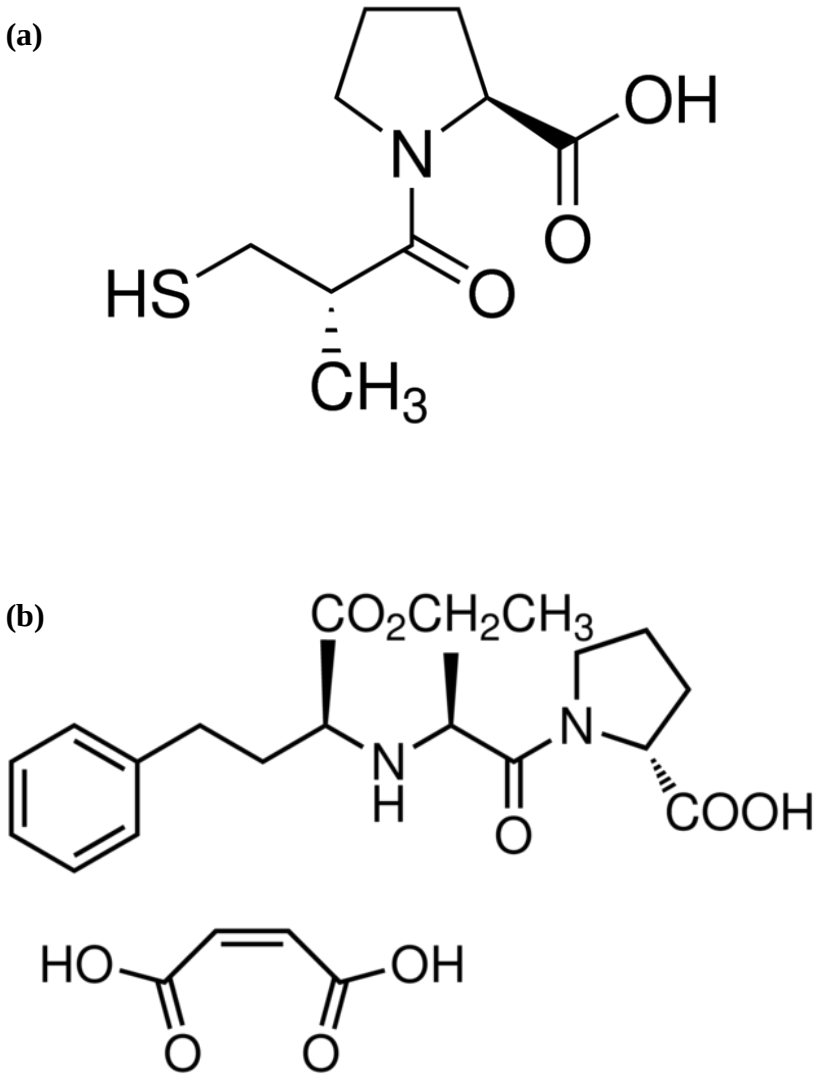


Figure 1.3. Chemical structures of the ACE inhibitors (a) captopril and (b) enalapril maleate. Captopril is the smallest ACE inhibitor structurally, and one of only a few to contain a sulfydryl group. Enalapril is much larger structurally, and belongs to the group of dicarboxylate-containing ACE inhibitors, of which there are several members. Structures downloaded from Sigma-Aldrich (*www.sigmaaldrich.com*)

1.7 Aims of the Present Study

All previous studies on the ability of ACE inhibitors to inhibit MMPs have focussed on the inhibition of MMP-2 and MMP-9, and to the authors knowledge no attempts have been made to study their efficacy in inhibiting other MMPs. Furthermore, no previous study has investigated the ability of ACE inhibitors to control the rate at which collagen based scaffolds or tissues degrade, which is primarily mediated through the collagenolytic action of interstitial collagenase (MMP-1). The loss of mechanical strength and stiffness of collagen-based scaffolds through cell-mediated enzymatic degradation is a serious problem, and the development of potent yet safe MMP inhibitors could provide a solution. Since ACE inhibitors are a common and physiologically safe drug that have been successfully used clinically for many years, it would be beneficial if their potential for MMP inhibition could be further exploited in the field of tissue engineering. With these points in mind, the aims of the present study are as follows:

1. To determine whether the ACE inhibitors captopril and enalapril are able to inhibit collagen degradation by collagenase at concentrations that are non-toxic to cells in surrounding tissues;

2. To develop an effective novel technique for the mechanical characterisation of the time-dependent properties of collagen hydrogel scaffolds;

   and, assuming the success of the previous two aims;

3. To attempt to exploit the collagenase inhibiting properties of the selected ACE inhibitors to regulate the loss in mechanical stiffness experienced by anchored cell-seeded collagen hydrogels.

Chapter 2

Materials and Methods: General

2 Materials and Methods: General

2.1 Preparation of Type I Collagen

2.1.1 Isolation of Collagen from Rat Tail Tendon

Collagen was extracted from rat tail tendons to produce soluble collagen according to the method of Elsdale and Bard (1972).

Dialysis tubing was cut into approximately 50 cm lengths and pre-treated by simmering for 1 hour in 50% (v/v) ethanol, followed by simmering twice for 1 hour in 10 mM $NaHCO_3$/1 mM ethylenediaminetetraacetic acid (EDTA). The tubing was finally simmered twice for 1 hour in sterile distilled water (dH_2O) and stored at 4 ºC in dH_2O until required.

Scalpels and forceps were autoclaved in advance to ensure sterility. Rat tails were sprayed with alcohol, snapped in the middle and cut into two sections of roughly equal length using a scalpel. An incision was made down the length of the tail so that the skin could be peeled off. The tendon bundles were then extracted from the tails using sterile forceps and placed in dH_2O. Once all the tendon bundles had been extracted the tendons in dH_2O were poured into gauze swabs (Type 13 light, Clini Supplies Ltd., UK) and squeezed to remove the majority of the water content. The tendons were then extracted into 0.5 M acetic acid at approximately 6 to 8 tails per 500 ml bottle and allowed to dissolve at 4 ºC for 48 h. The solubilised tendon solution was then filtered through sterile gauze to remove any remaining components that had not dissolved.

The previously prepared dialysis tubing was rinsed in 0.1 × Dulbecco's Modified Eagle's Medium (DMEM) and double knotted at one end. The solubilised tendon solution was poured into the dialysis tubing, leaving around 7-8 cm at the end of the tubing for air, and double knotted again. The tendon solution was then dialysed twice against 0.1 × DMEM at 4 ºC for 24 h. Following dialysis the solution was loaded into sterile centrifuge bottles (250 ml) and sterilised by centrifugation at 10 000 rpm and 6 ºC for 2 h. The supernatant was transferred into sterile bottles using sterile pipettes.

To calculate the concentration of collagen in the solution (mg/ml) 1 ml of the solution was transferred into each of 3 pre-weighed 100 mm Petri dishes which were then incubated at 37 ºC to allow the collagen to dry out. The Petri dishes were then weighed again, and the average difference in weight was taken as the collagen concentration.

To check the sterility of the collagen solutions 1 ml was taken from each bottle and incubated at 37 ºC in Sabourauds medium and in brain heart infusion for at least 72 h.

2.1.2 Preparation of Collagen Hydrogels

0.2-0.4% (w/v) collagen hydrogels were prepared by mixing collagen solution, 10 × DMEM and 0.4 M NaOH (2:1), and 1/1000 (v/v) acetic acid. The DMEM/NaOH was always added in the ratio 1:9, while the proportions of the other constituents were calculated according to the collagen concentration in the solution and the required collagen concentration of the gels. As an example, to make 10 ml of 0.3% (w/v) collagen gel using dissolved collagen at a concentration of 5.77 mg/ml the following volumes would be required: 5.2 ml dissolved collagen solution, 1 ml of 10 × DMEM/0.4 M NaOH (2:1), and then 3.8 ml of 1/1000 acetic acid to make the volume up to the total 10 ml.

The three ingredients were mixed in a sterile beaker and the pH adjusted by dropwise addition of 1 M NaOH while constantly swirling to mix. In order for the solution to gel the pH must be in the region of 8.0-8.5, and this is achieved by using the colour of the solution as an indicator (DMEM contains Phenol Red which turns a pink-purple colour at pH > 8.0). Once the solution had turned pink-purple it was quickly pipetted into appropriate culture dishes (taking care to avoid creating bubbles) and allowed to set for 2 h at room temperature in a laminar flow hood. Gels were then washed twice with DMEM, incubating each wash at 37 ºC for at least 20 min to allow the collagen to become fully polymerised.

2.2 Mammalian Cell Culture

2.2.1 Cell Line Used

Immortalised mouse fibroblast (3T3) cells, established from the National Institutes of Health Swiss mouse embryo (Todaro & Green, 1963) were used for all cell-based studies over the course of this research.

2.2.2 Maintaining a Cell Line

Cells were cultured in DMEM (Catalogue number BE12-604F, Lonza) supplemented with 10% foetal bovine serum (Catalogue number A15-101, PAA Laboratories), 50 units/ml penicillin, 50 µg/ml streptomycin, and 1% (v/v) non-essential amino acids. Cells were maintained as monolayers in 25 or 75 cm^2 flasks in an incubator (37 ºC and 5% CO_2 in air) and were routinely passaged every 3-4 days using a split ratio of between 1:15 and 1:25.

To split the cells, the culture medium was first discarded and the cell monolayers washed twice with versene (0.02% w/v). A small amount of trypsin (0.05% w/v) in versene was then added, ensuring that the entire cell monolayer was completely covered. When the cells had become fully detached from the flask, fresh DMEM was added and the cell suspension was pipetted up and down repeatedly using a sterile pipette in order that any larger clumps of cells could be broken down. A proportion of the cells in DMEM was then transferred to a new flask and topped up with fresh medium to achieve the desired spilt ratio.

2.2.3 Seeding Cells for Experiments

When setting up experiments cells were treated in the same manner as detailed in **section 2.2.2**, but instead of splitting the cell suspension into flasks, the cells were retained in a sterile universal until required and an estimate of the cell number per ml was made microscopically using a haemocytometer. The amount of cells required to

achieve a predetermined seeding density was calculated, and cells were seeded into tissue culture dishes or onto collagen hydrogels as required.

2.3 Preparation of Collagenases

2.3.1 Preparation of Mammalian Collagenase

Reagent A: 50 mM 2-(Tris(hydroxymethyl)methylamino)ethane-1-sulphonic acid (TES) buffer with 0.36 mM Calcium chloride pH 7.4.

A cell homogenate was used as a source of mammalian collagenase. For this 3T3 cells were first cultured to confluency in 75 cm^2 flasks. Cell monolayers were then washed twice with 5 ml cold phosphate buffered saline (PBS) pH 7.4. The cells were scraped in 1.5 ml *Reagent A*, and the resulting mixture was homogenised using 7 strokes of a motor driven Teflon-glass homogeniser before being stored in 1 ml aliquots at -70 °C until required. For certain batches cells were incubated in growth medium containing 1 mM concentrations of each of the ACE inhibitor drugs for 72 h before they were homogenised. The method for total cell protein measurement of the homogenate samples is outlined in **section 2.4**.

2.3.2 Preparation of Bacterial Collagenase

Type II bacterial collagenase (240 units/mg) prepared from *Clostridium histolyticum* was purchased from Invitrogen™, UK (Catalogue number 17101-015, lot number 618705). Collagenase was dissolved in *Reagent A* or dH_2O as appropriate to give a working solution of 0.2 mg/ml (≈ 50 units/ml), and was used immediately or frozen at -80 ºC until required.

Bacterial collagenase is a crude preparation containing clostridiopeptidase A and a number of other proteases, polysaccharidases and lipases (Mookhtiar & Van Wart, 1992; Worthington & Worthington, 2011), where one unit is said to liberate 1 μM of L-leucine equivalents from collagen in 5 hours at 37 °C (Invitrogen™, 2011).

2.4 Total Cell Protein Measurement

Reagent B: 0.01% w/v $CuSO_4$ with 0.01% w/v Na-K tartrate and 2% w/v Na_2CO_3.

Reagent C: 25% v/v Folin and Ciocalteu's Phenol Reagent in dH_2O.

To determine the total protein content of prepared cell homogenates, and thus estimate the likely ratio of collagenolytic proteins between homogenate samples, the method of Lowry *et al* (1951) was employed. Bovine Serum Albumin (BSA) was used as the protein standard, and a 200 µg/ml stock solution of BSA in 0.5 M NaOH was prepared and stored at -20 ºC. 1 ml of each BSA protein standard was prepared immediately before use by further diluting the stock solution in 0.5 M NaOH to achieve final standard concentrations of 0, 25, 50, 100, 150 and 200 µg/ml. Homogenate samples were prepared by taking 50 µl of each sample into a test tube and diluting with 950 µl NaOH.

5 ml of *Reagent B* was added to all of the standards and samples, mixed well and incubated at room temperature for 10 min. 0.5 ml of *Reagent C* was then added to each standard and sample and immediately mixed thoroughly before incubating at room temperature for approximately 1 h. The absorbance of the protein standards was read against dH_2O at 725 nm to produce a standard curve. The absorbance of the samples was then also read at 725 nm and the protein content estimated using the standard curve. Absorbance was read using a Shimadzu UV-2401PC two-channel spectrophotometer.

Chapter 3

Collagen Degradation Studies

3 Collagen Degradation Studies

3.1 Introduction

3.1.1 Sources of Collagenase

Two different sources of collagenase were used in the present study to assess the ability of the ACE inhibitors enalapril and captopril to inhibit the proteolytic degradation of collagen-based substrates. Cell homogenate from 3T3 fibroblasts was one of these sources, and was considered appropriate since fibroblasts are the natural producers of ECM-degrading proteins in mammals and are known to play a critical role in tissue remodelling (Grinnell, 1994). An ability to inhibit mammalian collagenase would indicate a potential for manipulating the rate at which cells are able to remodel and reshape a collagen-based substrate.

The other source of collagenase was bacterial collagenase from *clostridium histolyticum*, which is a crude preparation containing the collagen-cleaving clostridiopeptidase A, and a number of other less specific proteases, polysaccharidases and lipases (Mookhtiar & Van Wart, 1992; Worthington & Worthington, 2011). Although not produced natively by mammalian cells, bacterial collagenases have been implicated in the progression of many human diseases (Harrington, 1996), and have successfully been used for many years to treat necrotic skin conditions (Mandl, 1982; SANTYL®, 2011). To date, tissue engineered collagen has most commonly achieved clinical success when used for the treatment of necrotic conditions such as venous leg and diabetic foot ulcers (Allie *et al*, 2004; Falanga, 1998; Kirsner, 1998). The study of collagen degradation by bacterial collagenase is therefore highly relevant to the field of tissue engineering, since its inhibition may ultimately allow for tissue engineered collagen-based constructs to be used in combination with collagenase-based treatments, which could in turn lead to improved rates of repair and regeneration of damaged tissues when compared with the application of a single treatment alone. Furthermore, since bacterial collagenase has such a broad substrate specificity and high activity (compared with mammalian collagenase) (Mookhtiar & Van Wart, 1992), it was also hypothesised that its inhibition by ACE inhibitors could be indicative of a propensity for inhibiting a wide

range of proteolytic MMPs, and that any inhibitory effect could potentially be even greater on mammalian collagenases.

3.1.2 Collagen Degradation Assay

When examining the potential of specific substances or agents to inhibit the activity of collagenase and ultimately to alter the rate of collagen degradation, it is important to utilise methods that can not only detect the presence of collagen itself, but also the amount of enzymatic degradation that the collagen substrate is undergoing. The standard protocol for measuring collagen degradation and quantifying collagenase activity (Sigma, EC 3.4.24.3) is a modification of the procedure of Mandl *et al* (1953), where collagenase is incubated with native collagen and the extent of collagen breakdown is quantified using the colourimetric ninhydrin method (Moore & Stein, 1948). This method uses an L-leucine standard curve to determine the concentration of amino acids equivalent to leucine liberated from the digestion of collagen. As this protocol is relatively straightforward it was chosen for the initial collagen digestion experiments. However, inconclusive results prompted a shift to electrophoresis-based assays (see below).

3.1.3 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE is a widely used technique for separating proteins of interest according to their molecular masses, or more specifically their electrophoretic mobility. It has been shown that the separation of native proteins on polyacrylamide gels is not only dependent on their charge, but is also strongly correlated with the size of the molecules (Davis, 1964; Ornstein, 1964). A major breakthrough in protein analysis came when Shapiro *et al* (1967) first described the effect of SDS (a powerful protein dissociating detergent) in improving polyacrylamide gel electrophoresis of proteins. Weber & Osborn (1969) then went on to establish SDS-PAGE using a continuous buffer system as a reliable and straightforward method for the estimation of

molecular masses of protein subunits by demonstrating the relationship between the electrophoretic mobilities of proteins and the known molecular weights of their polypeptide chains. However, it was the discontinuous buffer system originally described by Laemmli (1970) that became the most commonly used type of SDS-PAGE.

Although all proteins have their own intrinsic charge, the majority of proteins bind SDS in a constant weight ratio of 1.4 g/g of polypeptide (Reynolds & Tanford, 1970). As a result the negative charges contributed by SDS effectively mask the intrinsic charge of the polypeptides. Consequently, the SDS-polypeptide complexes essentially possess identical charge densities, meaning that they hold the same net negative charge per unit length. The effect of this uniform charge density is that the mobility of these proteins within polyacrylamide gels occurs only as a function of their molecular weights, and the effect of differences in their mass charge ratios are negligible. It has also become customary to run SDS-PAGE under reducing conditions, where proteins of interest are heated to near boiling in the presence of a reducing agent (commonly 2-mercaptoethanol), which further denatures proteins by cleaving disulfide bonds and ensures optimal binding of SDS to the denatured polypeptides, allowing for separation by size and not by shape (Laemmli, 1970).

All types of collagen are fabricated from combinations of polypeptide strands of different molecular weights, and therefore SDS-PAGE is a suitable technique for detecting not only the presence of collagen, but also its purity and the sizes of individual molecules. SDS-PAGE can also be considered semi-quantitative in that the size and intensity of individual bands can be related to the amount of the specific polypeptides they represent (Miles & Saul, 2005), meaning that proteins having undergone degradation should result in smaller, less intense bands than the equivalent bands for proteins that have not been degraded. Furthermore, the degradation of collagen by collagenase involves the cleavage of large collagen monomers into smaller subunits, and many of these subunits may also be detectable via SDS-PAGE. It was therefore decided that SDS-PAGE would be a suitable technique for assessing collagenolytic breakdown of collagen, and the potential inhibition of this degradation.

3.1.4 Substrate Zymography

Substrate zymography is an electrophoretic technique based on SDS-PAGE, that also includes a substrate of interest co-polymerised with the polyacrylamide gel for the purpose of detecting enzyme activity (Lantz & Ciborowski, 1994). The technique can be used qualitatively to identify MMPs by the degradation of their preferential substrate and by their molecular weight. Zymography has also been presented as a quantitative technique, as it has been shown that the degree of substrate digestion within the zymogram is directly proportional to the amount of enzyme loaded (Kleiner & Stetlerstevenson, 1994).

Although there are several types of substrate zymography, the original and most common form is gelatin zymography (Snoek-van Beurden & Von den Hoff, 2005). However, all substrate zymography techniques are similar except that the substrate of interest differs depending on the type of MMPs to be detected. Proteins are separated by electrophoresis under denaturing (SDS), non-reducing (without a reducing agent or boiling) conditions, in order that enzymes retain their native states and therefore also their proteolytic activities. During electrophoresis the SDS actually causes the MMPs to denature and become inactive, but a subsequent wash causes exchange of the SDS with Triton X-100, causing the enzymes to partially renature and recover their activity (Heussen & Dowdle, 1980). The gel is then incubated in an appropriate activation buffer, and the concentrated, renatured MMPs within the gel will digest the substrate. Following incubation the gel is stained with Coomassie Blue, and areas of MMP activity are detected as clear bands against a dark blue background of undegraded substrate. These clear bands representing areas of MMP activity can then be quantified by densitometry (Frederick Woessner Jr, 1995).

Although it was initially thought that the incorporation of native collagen fibrils into polyacrylamide gels would be unsuitable for substrate zymography due to their complicated structure, it appears that SDS disrupts much of the fibrillar organisation of collagen, allowing proteins to run into the gel (Gogly *et al*, 1998). Furthermore, detergent exchange of SDS with Triton X-100 during the incubation stage is thought to restore part of the original triple-helical conformation of collagen, allowing its degradation by collagenase (Snoek-van Beurden & Von den Hoff, 2005). In fact,

Gogly *et al* (1998) showed collagen substrate zymography to be a highly sensitive and specific technique for determining low levels of interstitial collagenase, and also suggest the technique to be highly quantifiable by detailing a method that relates the size and optical intensity of degradation bands to the amount of collagenase in the sample. Nevertheless, collagen zymograms are known to be technically difficult and are not widely used (Troeberg & Nagase, 2001).

It is also of note that gelatin substrate zymography has previously been used successfully in determining the potential for captopril to inhibit gelatinase (Sorbi *et al*, 1993), although it was suggested that the required concentrations for inhibition may not reflect the real inhibitory capacity of captopril as high concentrations are required to penetrate the polyacrylamide-gelatin complex. It appears likely that collagen substrate zymography should be able to detect inhibition of collagenase by ACE inhibitors using a similar methodology. To this end a method was developed and optimised which could be used to analyse collagen degradation, and its inhibition.

3.2 Materials and Methods

3.2.1 Materials

Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich, Gillingham, Dorset, UK. Collagen was prepared in-house from rat tail tendons according to the method of Elsdale and Bard (1972), as described in section **2.1.1**. Preparation of collagenases are described in section **2.3**.

3.2.2 Collagen Digestion Assays

Reagent A: 50 mM TES buffer with 0.36 mM Calcium chloride pH 7.4.

Collagen was extracted from rat-tail tendons to produce soluble collagen according to the method of Elsdale and Bard (1972) at concentrations of between 4 and 6

mg/ml in 0.1% (v/v) acetic acid. The collagen solution was pipetted into universals at appropriate volumes and the acid allowed to evaporate in order that 10 mg of the collagen solute remained. Captopril and enalapril at required concentrations were obtained by dissolving the drugs in Reagent A. A maximum of 2 ml of Reagent A was added to the collagen samples and they were incubated at 37 °C until equilibrated. The volume of buffer was adjusted accordingly for samples where drugs, sources of collagenase, or both were to be added. Once equilibrated 20 or 100 µl of bacterial collagenase solution (1 or 5 units approx.) or 3T3 cell homogenate containing approximately 1.5 mg homogenised proteins, prepared according to **section 2.3.1**, as well as any drugs were added to the appropriate samples. The samples were mixed well and incubated at 37 °C. The incubation time was 5 hours, with the universals being shaken for 10-15 seconds at approximately 1.5 and 3.5 hours. The resulting solutions were then used with the ninhydrin colour development assay or collected into 1 ml aliquots and stored at -70 °C for use with SDS-PAGE to determine the extent of breakdown of the collagen.

3.2.2.1 SDS-PAGE

SDS-PAGE was carried out using a Fisherbrand Vertical Electrophoresis Unit (Model FTV100) mini-gel apparatus, according to the manufacturer's instructions, with 10% (w/v) bis-acrylamide resolving gels. Resolving gels were cast by mixing acrylamide/bis-acrylamide (40%), 1.5 M Tris pH 8.8, 1.5% ammonium persulfate (APS) and dH_2O at a ratio of 5:5:9:1 before adding 17.25 µl Tetramethylethylenediamine (TEMED). A 4% (w/v) bis-acrylamide stacking gel was cast over the resolving gel in order to concentrate the samples and was prepared by mixing acrylamide/bis-acrylamide (40%), 0.5 M Tris pH 6.8, 1.5% APS and dH_2O at a ratio of 2:5:1:12 before adding 10 µl TEMED.

Samples were prepared at 1 mg/ml protein in Laemmli buffer (catalogue number S3401, Sigma) and boiled for 2 minutes in stoplock Eppendorf tubes before being loaded into the gels at 20 µg protein/well. Molecular weight markers were loaded at 3 µl/well where appropriate. Gels were then run at 50 mA (variable voltage) for

approximately 15 min to allow the samples to concentrate in the stacking gel, and then at 100-150 V (variable current) until the samples had run to within 3-4 mm of the bottom of the gel. Following the electrophoretic separation, proteins were visualised by staining in Coomassie Brilliant Blue R-250 solution (1.25 g Coomassie Brilliant Blue R-250, 454 ml methanol, 46 ml acetic acid, 500 ml dH_2O) for 1 hour on a shaker, and then destaining in a solution of methanol, acetic acid and dH_2O at a ratio of 3:1:6 until the background was clear. Alternatively gels were stained for at least 1 hour with Instant Blue (Triple Red Ltd., UK) when available, which allows for early visualisation of protein bands and removes the requirement of a destaining step.

Gels were imaged in greyscale with an Image Reader LAS-3000 or Canon CanoScan N670U.

3.2.2.2 SDS-PAGE Analysis

Image analysis (densitometry) was performed using ImageJ (National Institutes of Health, *http://rsbweb.nih.gov/ij/*) software package. The software was used to compare the sizes and intensities of individual protein bands using a method similar to that published by Miles & Saul (2009). The spectral densities of protein bands were plotted relative to the distance they had migrated down the gel, and the sizes of the resultant peaks representing individual bands were recorded and compared. Results were then compared by Analysis of Variance (ANOVA) followed by Dunnett's test (when comparing with a single control sample) or Tukey's range test (when comparing results with each other), with 95% confidence intervals. As the sizes and intensities of protein bands are only compared against each other (and not against a set of standards) this method can only be considered semi-quantitative.

3.2.2.3 Collagen Zymography

7.5, 10 or 20% (w/v) bis-acrylamide resolving gels were prepared to contain 0.05% collagen (0.5 mg/ml). For certain experiments the pH of the resolving gel solution (normal pH ~8.8) was altered to approximately pH 8 or 9.5 before gelation through dropwise addition of 1 M HCl or 1 M NaOH respectively. Samples were prepared by mixing collagenase with non-reducing Laemmli buffer (without the reducing agent β-mercaptoethanol) at an even ratio, and without heating. Samples were then loaded and gels were run using the conditions as for SDS-PAGE at 4 °C, 15 °C, or room temperature until the bromophenol blue tracking dye reached the bottom of the gel. After running the gels they were rinsed in dH_2O before being incubated in 2.5% (v/v) Triton X-100 (100 ml for one or two gels) with gentle agitation for 45 minutes at room temperature to remove the SDS. The gels were rinsed again in dH_2O before being incubated for 4 or 18 hours at 37 °C in 50 ml zymogram developing buffer (100 mM Tris-HCl, 5 mM $CaCl_2$, 0.02% Brij-35, pH 8.0) to allow active enzymes to degrade the substrate, with enalapril, captopril or EDTA (a known collagenase inhibitor (Gogly *et al*, 1998)) at appropriate concentrations being added to the developing buffers of selected gels. Gels were then rinsed again in dH_2O before being stained for approximately 30 minutes in Coomassie Brilliant Blue R-250 solution. Gels were then placed in dH_2O and areas of proteinase activity were evident as clear (unstained) bands against a dark blue background. Gels were imaged in greyscale with a Canon CanoScan N670U.

3.2.2.4 Collagen Zymography Analysis

Image analysis (densitometry) was performed using the ImageJ (National Institutes of Health, *http://rsbweb.nih.gov/ij/*) software package and a methodology similar to that specified by Gogly *et al* (1998). The software was used to compare the sizes and intensities of individual bands of enzymatic degradation, the size of each band being multiplied by its average pixel intensity to obtain values that could be compared in a semi-quantitative approach. Results were compared by ANOVA followed by Dunnett's test with respect to untreated zymograms (95% confidence intervals).

3.3 Results

3.3.1 Collagen Zymography

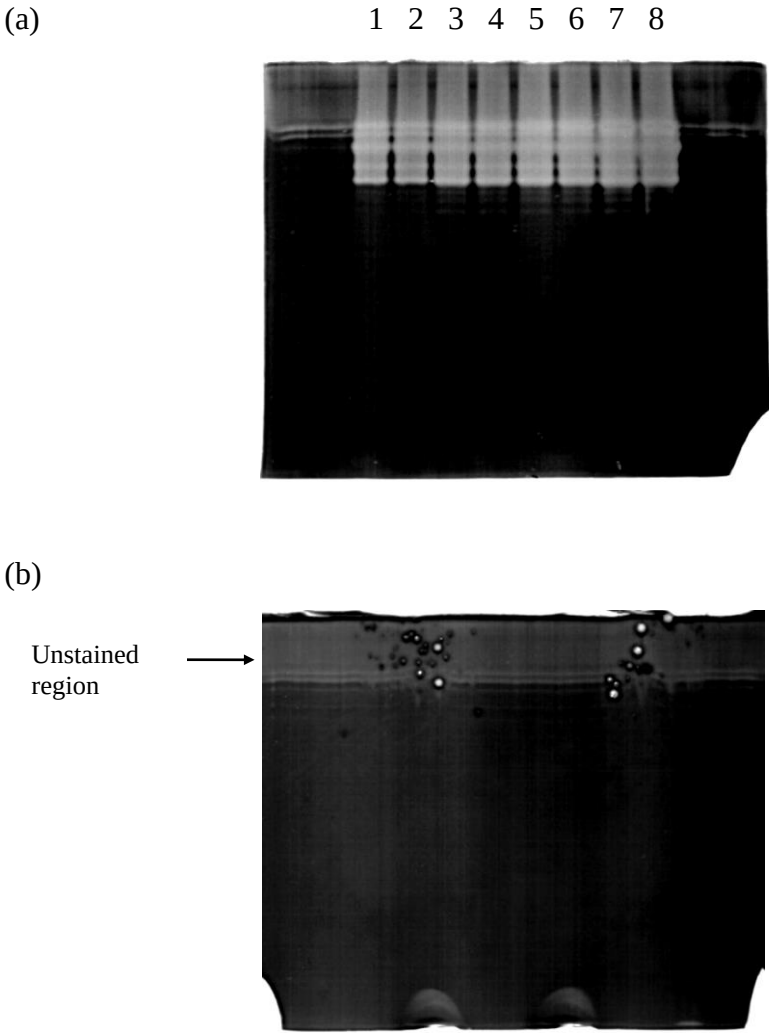


Figure 3.1. Zymography gel images from initial validation studies. (a) Lanes 1 and 2, 3 and 4, 5 and 6, 7 and 8 are 0.025, 0.05, 0.1, and 0.2 units bacterial collagenase respectively. (b) Blank zymogram with no collagenase. Gels were run at 125 V for 2.5 hours at room temperature and then incubated for 18 hours at 37 °C in zymogram developing buffer.

Initial zymography studies undertaken according to the method of Gogly *et al* (1998) suggested the technique may be viable, with clearly observable lysis bands in the lanes containing collagenase *(Fig. 3.1(a))*, though the areas of the bands were too large to analyze individually as they had merged and were overlapping. There was

also a large unstained area at the top of the zymograms that was still present in gels that had not been loaded with any collagenase *(Fig.3.1(b))*.

Although multiple bands were present for each amount of collagenase tested when zymograms were run at room temperature *(Fig. 3.1(a))*, only a single band was apparent for each lane when zymograms were run at 4 °C *(Fig. 3.2)*. The large unstained area at the top of the gel was also no longer present. Furthermore, a shorter incubation time resulted in smaller bands that did not overlap. Zymograms that were run at an intermediate temperature (approximately 15 °C) did not vary in appearance from those run at 4 °C (results not shown). Varying the voltage applied for electrophoretic separation did not alter the number of visible bands, although applying a 150 V potential resulted in less well defined bands *(Fig. 3.2 (b))* compared with those obtained from the application of a lower 100 V potential, which had a more regular shape *(Fig.3.2(a))*.

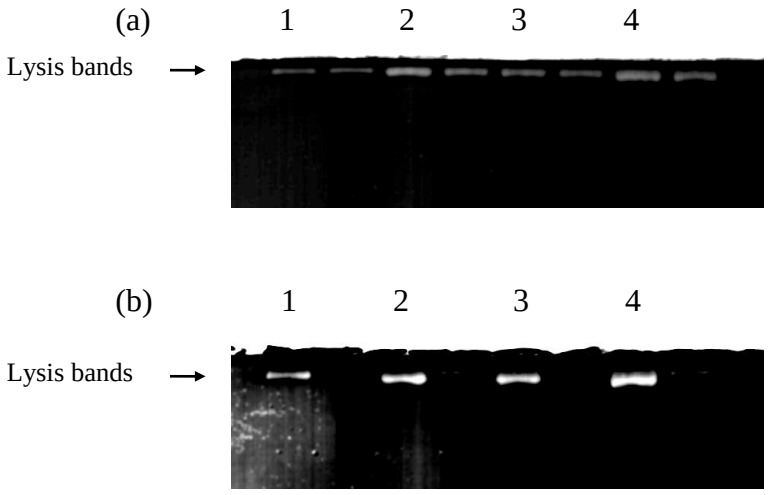


Figure 3.2. Zymography gel images from varying electrophoresis voltage studies. Lanes 1, 2, 3, and 4 are 0.025, 0.05, 0.1, and 0.2 units bacterial collagenase respectively. (a) Gel run at 100 V for 2.5 hours. (b) Gel run at 150 V for 2.5 hours. Both gels were run at 4 °C and then incubated for 4 hours at 37 °C in zymogram developing buffer.

Increasing the time for electrophoretic separation to 18 hours at 4 °C did not encourage the bands to migrate any further down the gel and did not result in more than a single band being observable in each lane *(Fig. 3.3)*. However, under these conditions altering the polyacrylamide content of the gels had some effect on the observed lysis bands, with 7.5% polyacrylamide gels *(Fig. 3.3(a))* showing bands that had migrated slightly further but were also less regular in shape and less well defined than those present on 10% polyacrylamide gels *(Fig. 3.3(b))*. Gels containing 20% polyacrylamide showed bands that had hardly migrated any distance into the gel and were very poorly defined and difficult to analyze *(Fig. 3.3(c))*.

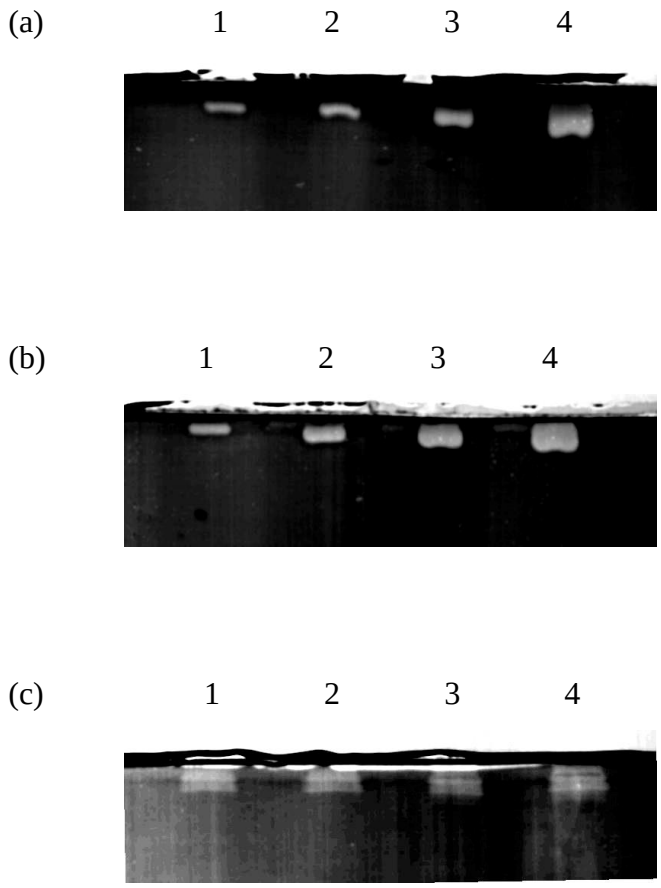


Figure 3.3. Zymography gel images from varying polyacrylamide concentration studies. Lanes 1, 2, 3, and 4 are 0.0625, 0.125, 0.25 and 0.5 units bacterial collagenase respectively. (a) 7.5% polyacrylamide gel. (b) 10% polyacrylamide gel. (c) 20% polyacrylamide gel. All three gels run at 125 V for 18 hours at 4 °C and then incubated for 4 hours at 37 °C in zymogram developing buffer.

Adjusting the pH of polyacrylamide gels before gelation did have a marked effect on the spread of lysis bands for gels run at room temperature *(Fig. 3.4(a, b))*. Lowering the pH to 8.0 resulted in a far greater spread of the degraded areas, with individual bands much better defined and no longer overlapping *(Fig. 3.4(a))*. However, the problem of background lysis was still very apparent, with the upper degradation

bands falling within the unstained area, and the unstained area itself having migrated further into the gel than for previous zymograms run at room temperature. Increasing the pH to 9.5 had the opposite effect, with all bands completely undistinguishable from each other and falling within the upper unstained area *(Fig. 3.4(b))*. Running gels at 4 °C in combination with lowering pH required loading gels with greater amounts of collagenase before lysis could be visualised, but still resulted in only a single lysis band being visible for each lane *(Fig. 3.4(c))*, though bands had migrated further into the gel and remained more uniform in shape than previous zymograms had shown at 4 °C.

Having tested and modified the technique it was decided that, despite the continued appearance of an unstained upper region, the results observed in *Fig. 3.4(a)* were the most usable due to the number of lysis bands observed, their spacing, and the clear correlation between the size and intensity of the bands and the amount of collagenase. Consequently, all subsequent zymograms would be set as 10% polyacrylamide gels at pH 8, run at 125 V for approximately 2.5 hours and at room temperature, and then incubated in zymogram developing buffer for 18 hours at 37 °C.

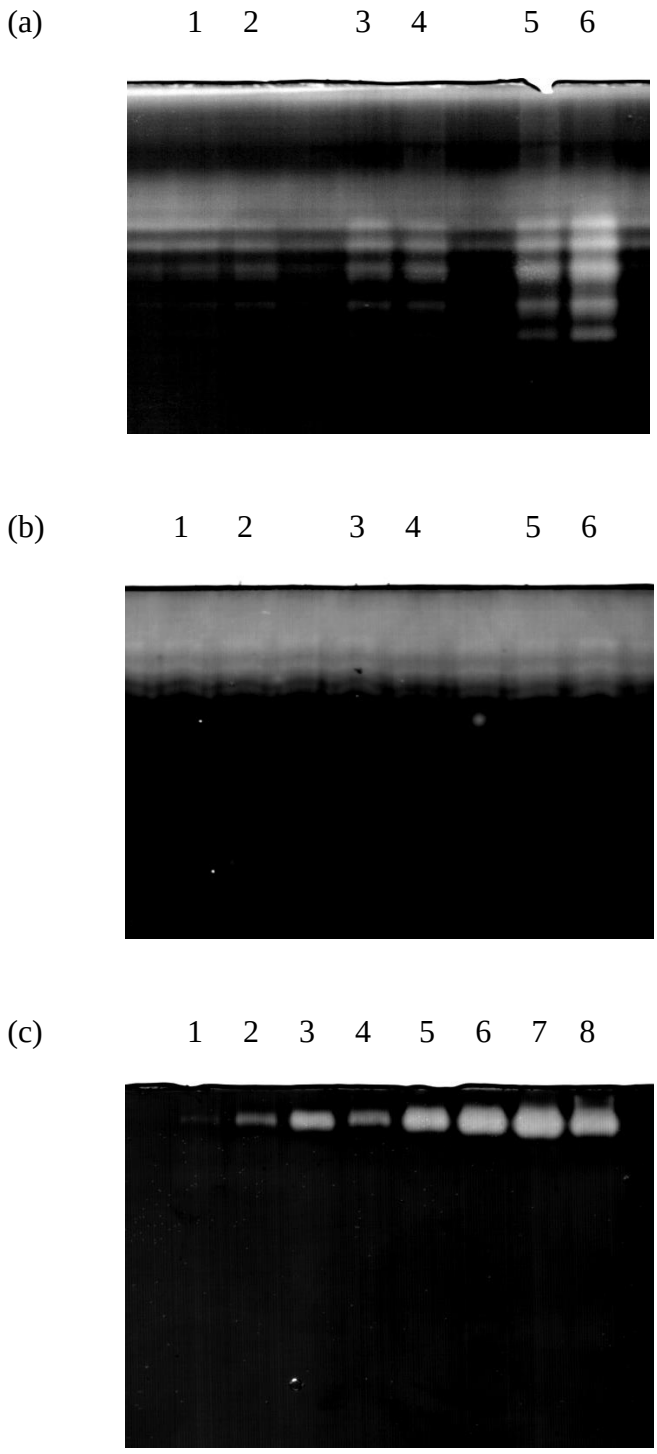


Figure 3.4. Zymography gel images from varying pH studies using 10% polyacrylamide gels. For (a) and (b) lanes 1, 2, 3, 4, 5 and 6 are 0.25, 0.5, 1, 2.5, 5 and 10 ng bacterial collagenase respectively. (a) pH 8 polyacrylamide gel. (b) pH 9.5 polyacrylamide gel. (c) Lanes 1, 2, 3, 4, 5, 6, 7, 8 are 12.5, 25, 50, 100, 200, 300, 400, 500 ng bacterial collagenase respectively, pH 8 polyacrylamide gel. (a) and (b) were run at 125 V for 2.5 hours at room temperature while (c) was run at 125 V for 4 hours at 4 °C. All three gels were incubated for 18 hours at 37 °C in zymogram developing buffer.

Zymograms incubated with untreated zymogram developing buffer *(Fig. 3.5(a))* clearly show increasing amounts of substrate degradation with increasing amounts of collagenase, with a total of five clearly visible bands at the higher collagenase concentrations. The zymograms shown in *Fig. 3.5 (b)* and *(c)* do not display any signs of collagenolytic degradation, implying that even EDTA 5 mM in the developing buffer is sufficient to completely inhibit the action of bacterial collagenase. The gels incubated with 5 and 10 mM enalapril, *Fig. 3.5 (d)* and *(e)* respectively, appear almost identical, with both showing marked inhibition of substrate degradation, with only the upper weight bands being slightly visible and only for higher amounts of collagenase. The gel incubated with 5 mM captopril *(Fig. 3.5(f))* looks similar to that of the negative control *(Fig. 3.5(a))*, with only small signs of inhibition for lower amounts of collagenase. However, the gel incubated with 10 mM captopril *(Fig. 3.5(g))* shows marked inhibition, with many bands not showing at all, and those that are present appearing much less intense than the equivalent bands of the negative control.

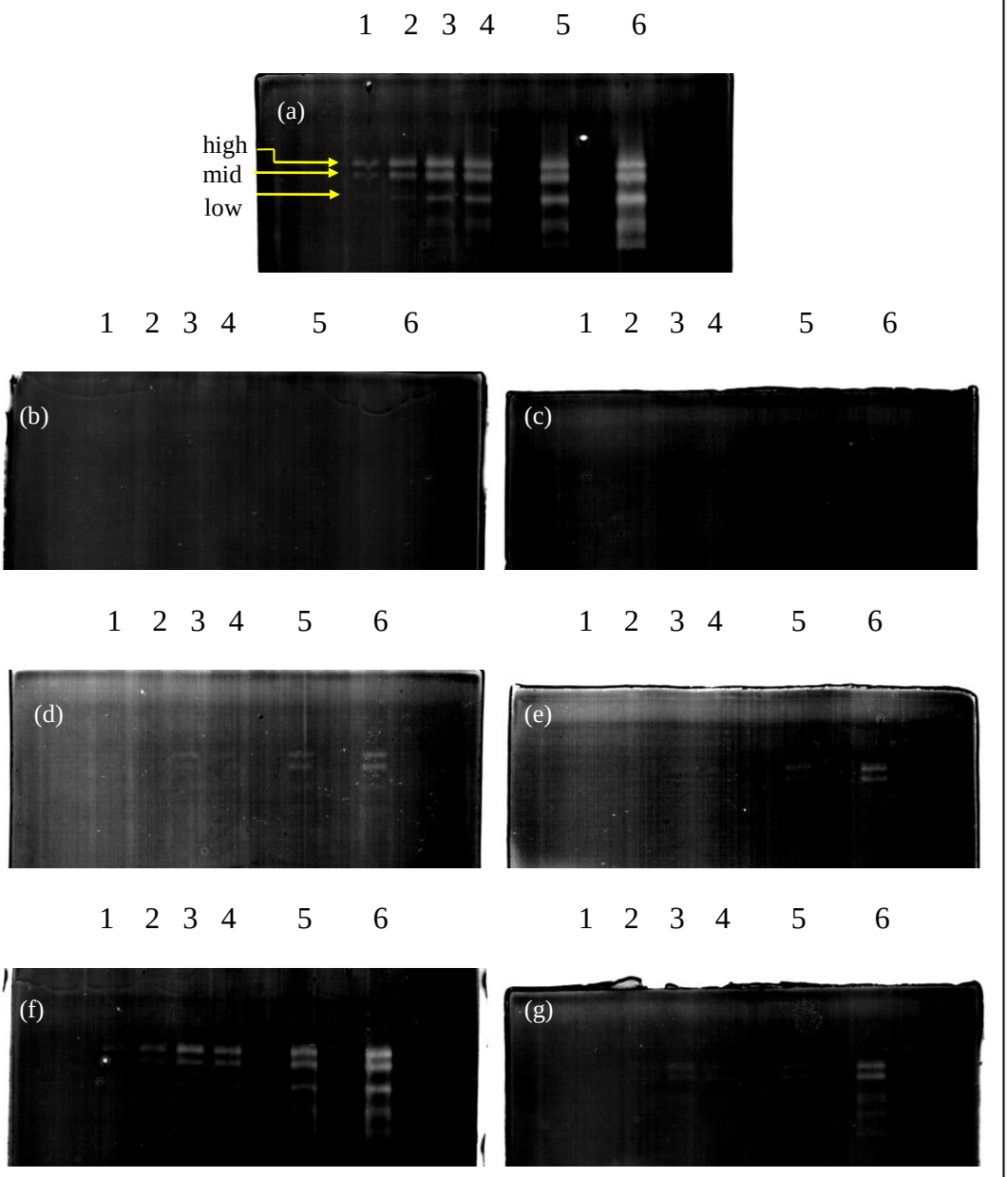
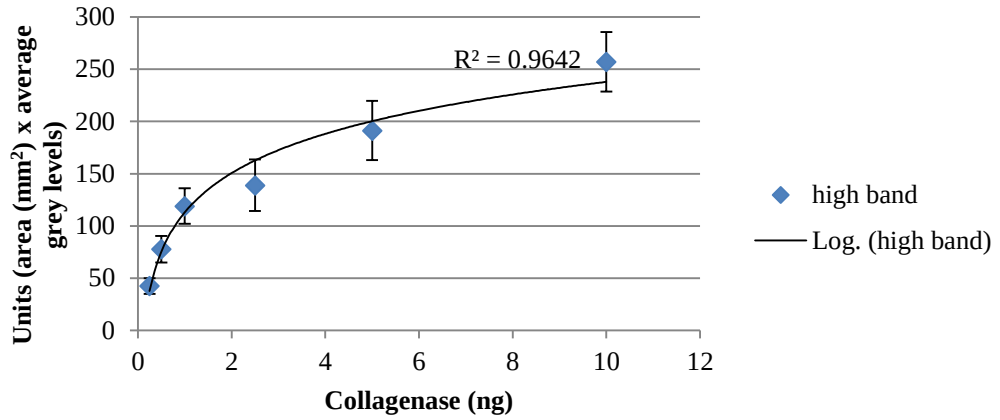


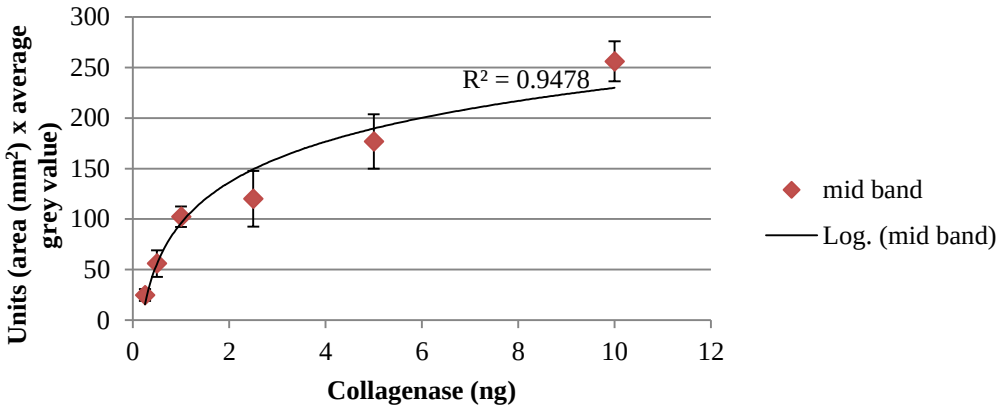
Figure 3.5. Images of zymograms from experiments with bacterial collagenase from *clostridium histolyticum*. For all zymograms lanes 1 to 6 are 0.25, 0.5, 1, 2.5, 5 and 10 ng collagenase respectively. (a) was incubated with untreated zymogram developing buffer, (b) and (c) with 5 and 10 mM EDTA respectively, (d) and (e) with 5 and 10 mM enalapril respectively, and (f) and (g) with 5 and 10 mM captopril respectively. Lysis bands for subsequent analysis *(Fig. 3.13, Fig.3.14)* are marked in (a). Time of incubation 18 hours.

The three highest molecular weight bands (*Fig. 3.5(a)* marked ‘high’, ‘mid’, ‘low’) were analysed further in order to ascertain relationships between the size and intensities of the bands and the amount of collagenase loaded into the gels, and hence further validate the method for use with collagenase from *clostridium histolyticum*. Lysis bands were quantified by image analysis, with collagenase activities expressed as Units = surface area (mm^2) x average grey levels of the lysis band. The two highest molecular weight bands show a similar logarithmic relationship with increasing collagenase concentration *(Fig. 3.6(a)* and *(b))*, with R^2 values of 0.96 and 0.95 respectively, indicating a strong goodness of fit. The lowest molecular weight band analysed also showed a linear relationship with an R^2 value of 0.99 *(Fig. 6(c))*, indicating an excellent goodness of fit.

(a)



(b)



(c)

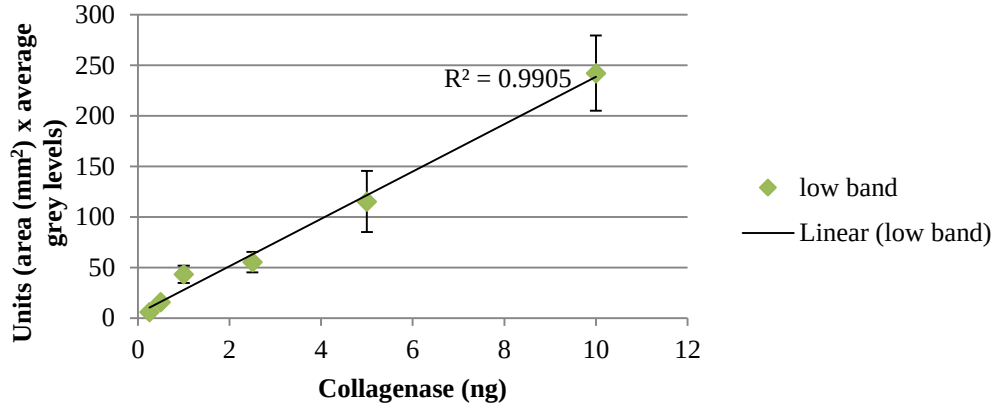


Figure 3.6. Quantification of collagenase from zymograms incubated with zymogram developing buffer using a range of 0.25-10 ng bacterial collagenase from *clostridium histolyticum* (see *Fig. 3.12(a)*). Time of incubation 18 hours. Lysis bands were quantified by image analysis, with collagenase activities expressed as Units = surface area (mm^2) x average grey levels of the lysis band. Results are mean ± SEM, n = 3.

Comparisons of the size and intensity of the three highest molecular weight bands following treatment with 5 and 10 ng collagenase across all zymograms confirmed the inhibitory effects of all tested substances. *Fig. 3.7* shows that EDTA at 5 mM (and also at 10 mM, not shown) completely inhibited the action of 5 and 10 ng collagenase to degrade the substrate ($p < 0.001$). All concentrations of captopril and enalapril tested showed marked inhibition of 5 and 10 ng collagenase, with enalapril appearing the more potent inhibitor *(Fig. 3.7)*. The average inhibition across all three bands tested for 5 mM captopril was 60.4 ± 14.6% ($p < 0.01$) and 57.9 ± 16.8% for 5 and 10 ng collagenase respectively, while at 10 mM captopril much greater inhibition was achieved with 93.1 ± 1.4% ($p < 0.001$) and 83.7 ± 5.6% ($p < 0.01$) inhibition respectively. Enalapril showed almost complete inhibition of collagenase at both 5 and 10 mM concentrations, with 93.4 ± 1.7% and 94.4 ± 0.9% inhibition at 5 mM, and 97.7 ± 1.5% and 95.5 ± 1.4% inhibition at 10 mM for 5 and 10 ng collagenase respectively. At both 5 and 10 mM, enalapril treated zymograms demonstrated statistically significant levels of collagenase inhibition compared to untreated controls at both 5 ng ($p < 0.001$) and 10 ng ($p < 0.01$) collagenase.

(a)

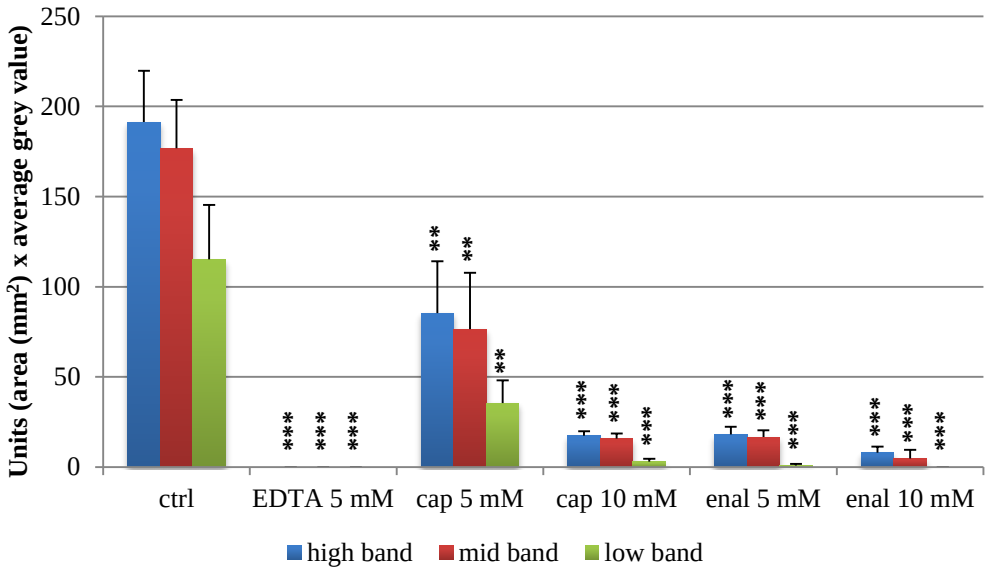
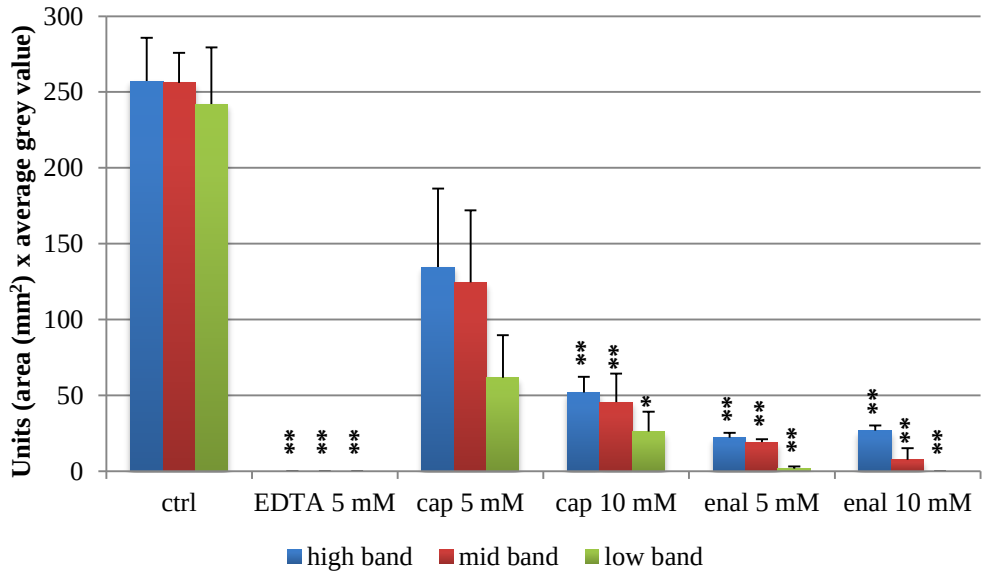


Figure 3.7. Comparative analysis of collagen zymograms for (a) 5 ng and (b) 10 ng bacterial collagenase from *clostridium histolyticum*. Time of incubation 18 hours, with potential inhibitors dissolved in incubation buffers where appropriate. Bars represent collagenase activities towards the three heaviest collagenolytic fragments and are expressed as Units = surface area (mm^2) x average grey levels of the lysis bands. 'Cap' = captopril, 'enal' = enalapril. Results are mean + SEM, n = 3. Results compared using ANOVA followed by Dunnett's test with respect to untreated zymograms ('ctrl'). Significant variations: ** $p < 0.01$, *** $p < 0.001$, 95% confidence.

(b)



3.3.2 SDS-PAGE

SDS-PAGE of collagen from rat tail tendon allowed for clear visualisation of the bands representing the various collagen molecules. The banding pattern was as expected for collagen type I, with bands appearing at approximately 140 kDa and 130 kDa representing the alpha-1 and alpha-2 chains respectively, and a band at approximately 270 kDa representing the dimeric form of collagen (β-band) *(Fig. 3.8)*. It should be noted that this upper band is in fact two separate bands (since the collagen monomers are different sizes, the resulting dimers are also different sizes), but for the purposes of analysis they have been treated as a single band as they are adjacent to each other and often merge where the signal is strong. There is also evidence of another band estimated to be around 400 kDa representing the trimeric form of collagen (γ-band), though its limited mobility meant that while its presence or absence could be noted, it was not suitable for quantitative analysis. Initial experiments using the rat tail collagen were inconclusive, with the main collagen bands appearing largely unaffected by the addition of mammalian cell homogenate and with lower degradation products being indiscernible from the cell homogenate itself *(Fig. 3.8)*.

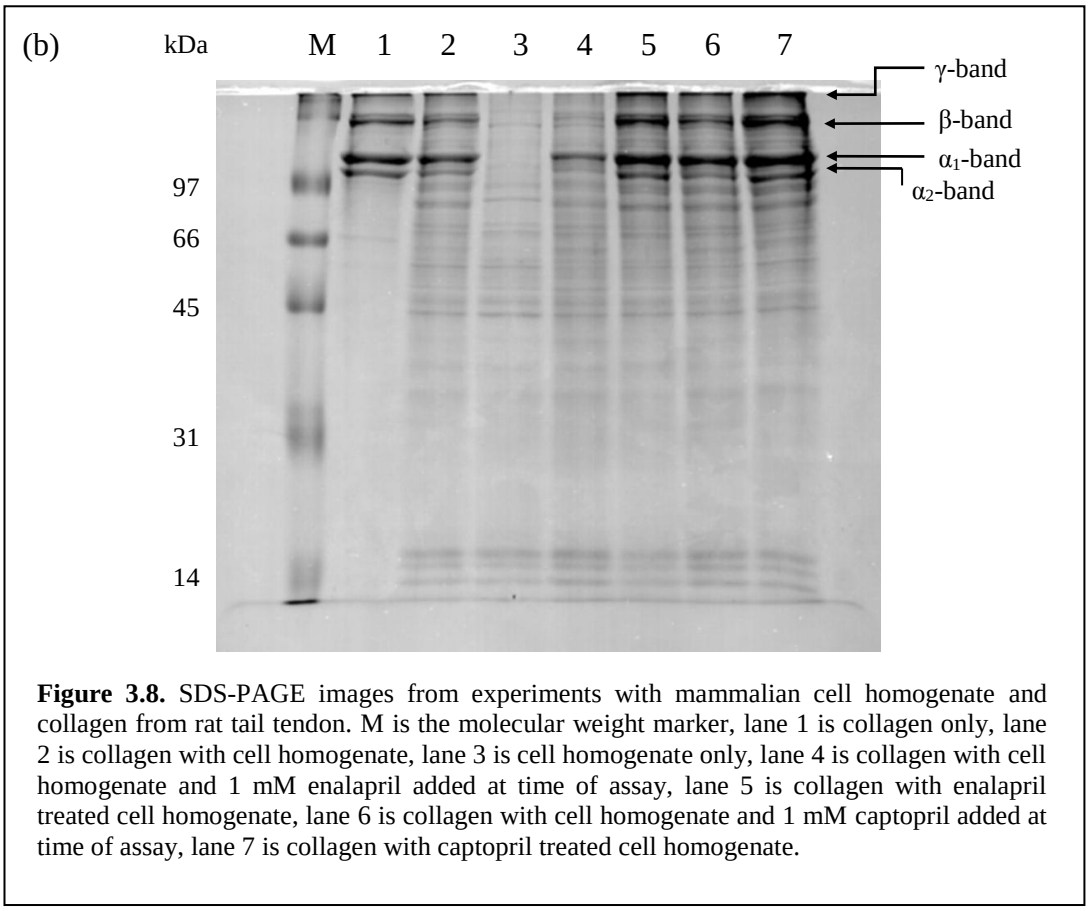


Figure 3.8. SDS-PAGE images from experiments with mammalian cell homogenate and collagen from rat tail tendon. M is the molecular weight marker, lane 1 is collagen only, lane 2 is collagen with cell homogenate, lane 3 is cell homogenate only, lane 4 is collagen with cell homogenate and 1 mM enalapril added at time of assay, lane 5 is collagen with enalapril treated cell homogenate, lane 6 is collagen with cell homogenate and 1 mM captopril added at time of assay, lane 7 is collagen with captopril treated cell homogenate.

A more concentrated cell homogenate did achieve degradation of the main collagen molecules as can be seen in *Fig. 3.9.* Very little breakdown of the main collagen protein bands was observed for the untreated collagen samples, whereas the equivalent protein bands are much less recognisable for the collagenase treated samples, and many more bands representing lower molecular weight fragments were observed. Samples treated with collagenase and various concentrations of captopril *(Fig. 3.9 (a))* or enalapril *(Fig. 3.9 (b))* added at the time of assay still show signs of breakdown, though the amount of degradation appears markedly less than that of the samples treated only with collagenase, and the main collagen bands still appear relatively intact in comparison. Furthermore, the amount of degradation of these upper protein bands appears to decrease with increasing concentrations of both drugs. The collagen bands appear to remain more intact for the enalapril treated samples *(Fig.3.9(b))* than those of the captopril treated samples *(Fig.3.9(a))*.

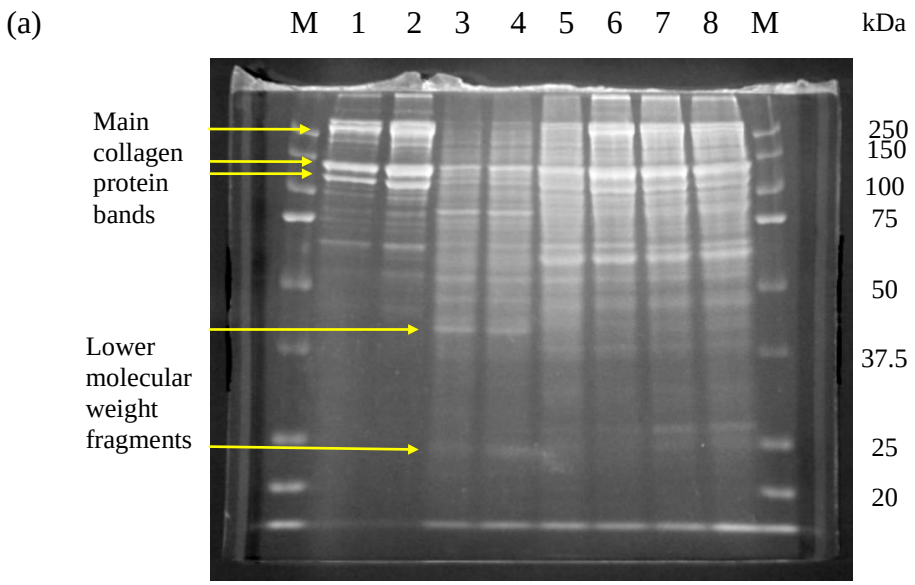
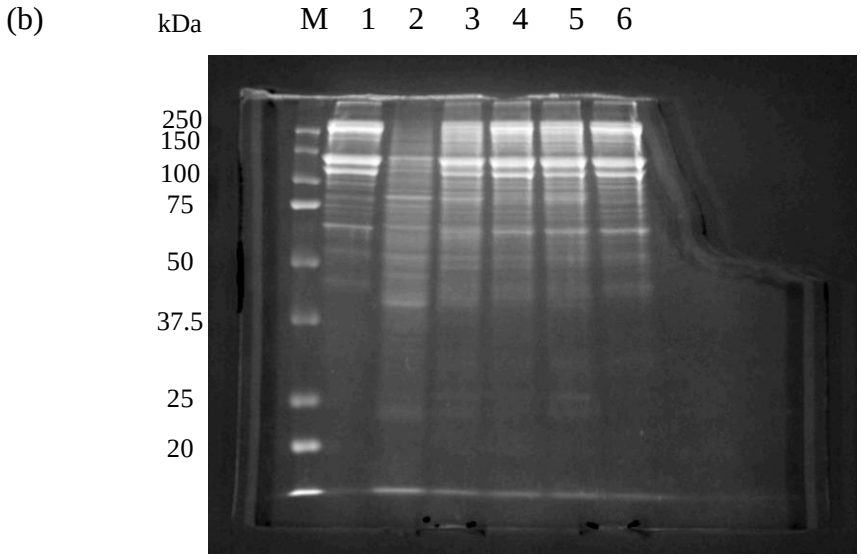


Figure 3.9. SDS-PAGE images from collagen degradation experiments using mammalian collagenase (from cell homogenate). For both images M is the molecular weight marker. (a) Lanes 1 and 2 are collagen, lanes 3 and 4 are collagen with mammalian collagenase, lanes 5 and 6 are collagen with collagenase and 5 mM captopril, lanes 7 and 8 are collagen with collagenase and 10 mM captopril. The positions of the three main collagen protein bands and some lower molecular weight fragments are marked with arrows. (b) Lane 1 is untreated collagen, lane 2 is collagen treated with mammalian collagenase. Lanes 3, 4, 5, and 6 are collagen treated with collagenase and enalapril at 1, 2.5, 5, and 10 mM respectively. For both sets of results the cell homogenate used contained approx. 2 mg protein, and ACE inhibitors were added at time of assay.



However, results obtained using cell homogenate were inconsistent and difficult to replicate since the proteolytic activities of the cell homogenate were not always sufficient to degrade the collagen substrate.

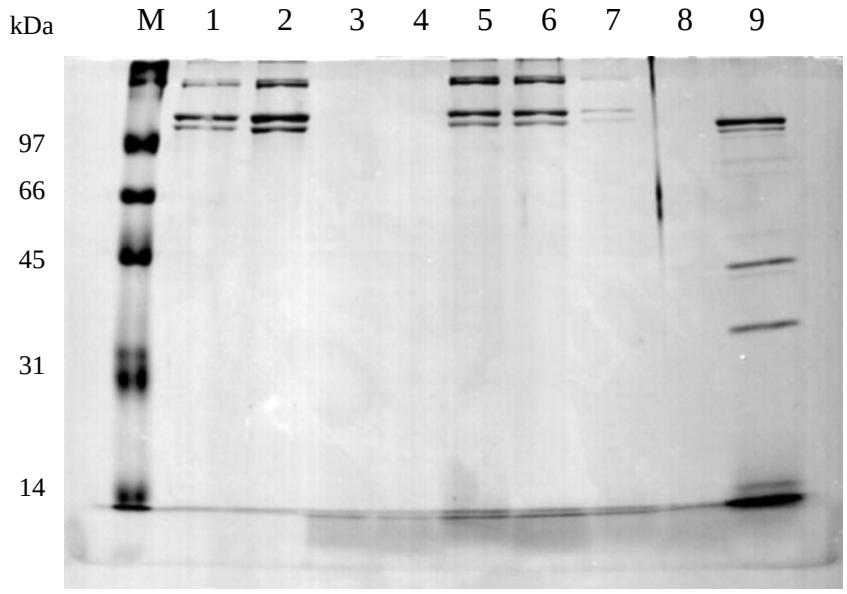


Figure 3.10. SDS-PAGE image from collagen degradation experiments with bacterial collagenase from *clostridium histolyticum*. Lane M is the molecular weight marker, lanes 1 and 2 are collagen, lane 3 is collagen with 1 unit collagenase, lane 4 is collagen with 5 units collagenase, lane 5 is collagen with 1 unit collagenase and enalapril 1 mM, lane 6 is collagen with 5 units collagenase and enalapril 1 mM, lane 7 is collagen with 1 unit collagenase and captopril 1 mM, lane 8 is collagen with 5 units collagenase and captopril 1 mM, lane 9 is collagenase only (1 mg/ml).

The use of bacterial collagenase instead of mammalian cell homogenate allowed for much clearer visualisation and quantification of collagenase inhibition. *Fig. 3.10* shows that collagen digested with either 1 or 5 units of collagenase (lanes 3 and 4, respectively) resulted in the complete disappearance of the main collagen bands, while the addition of enalapril 1 mM in each case showed these bands to remain almost fully intact (lanes 5 and 6). When captopril 1 mM was added to selected samples the bands remained clearly visible for the collagen sample digested with only 1 unit of collagenase, though they were only around half the size and intensity of the bands of the control sample, and were absent for the sample digested with 5

units collagenase (lanes 7 and 8) *(Fig. 3.10)*. Only the main collagen bands could be observed from these experiments and the only evidence of breakdown products further down the gel were some slightly darker areas at the very bottom of the gel in the lanes of collagenase treated samples. When collagenase was run by itself *(Fig. 3.10,* lane 9*)*, several protein fragments were observed, all of which were of lower molecular weight than even the smallest collagen fragment (although the largest collagenase fragments were very close in size to the smallest collagenase fragments), and none of which were present following its application to digest collagen *(Fig. 3.10,* lanes 1-8*)*.

For subsequent experiments the amount of collagenase added to appropriate samples was kept constant at 1 unit, with varying amounts of each ACE inhibitor being tested for collagenase inhibition. *Fig. 3.11* shows increasing intensities of the main collagen bands (marked "high", "mid" and "low" for analysis purposes) for increasing amounts of both inhibitors. Although there are marked signs of inhibition at 1 mM captopril, it is only at 2 mM captopril that the collagen bands appear fully intact *(Fig. 3.11(a))*. However, even at 0.5 mM enalapril the collagen bands are reasonably intact *(Fig. 3.11(b))*. At the higher ACE inhibitor concentrations tested the collagen bands appeared larger than and of similar intensities to those of the collagen-only controls for both captopril and enalapril *(Fig. 3.11)*.

These results were confirmed by image analysis (densitometry) of the three main collagen bands *(Fig. 3.12)*. Even at 0.25 mM captopril there was discernible inhibition of the high, mid, and low collagen bands with band intensities of 31.5 ± 23.6%, 22.8 ± 15.9%, and 30.1 ± 23.6% of the respective controls, compared with 14.9 ± 6.4%, 14.1 ± 6.0%, and 15.9 ± 5.7% for the samples treated with only collagenase *(Fig. 3.12(a))*. At 1 mM captopril there was little perceived difference from the lower concentrations of captopril tested with band intensities of 32.5 ± 14.9%, 31.2 ± 12.7%, and 26.3 ± 11.1% of the respective controls for the high, mid, and low bands *(Fig. 3.12(a))*. Only at 5 mM captopril were the collagen bands significantly larger ($p < 0.01$) than those of the samples treated only with collagenase, and more intense than those of the control samples at 229.7 ± 80.7%, 117.4 ± 36.7%, and 149.5 ± 42.6% for the high, mid, and low bands respectively.

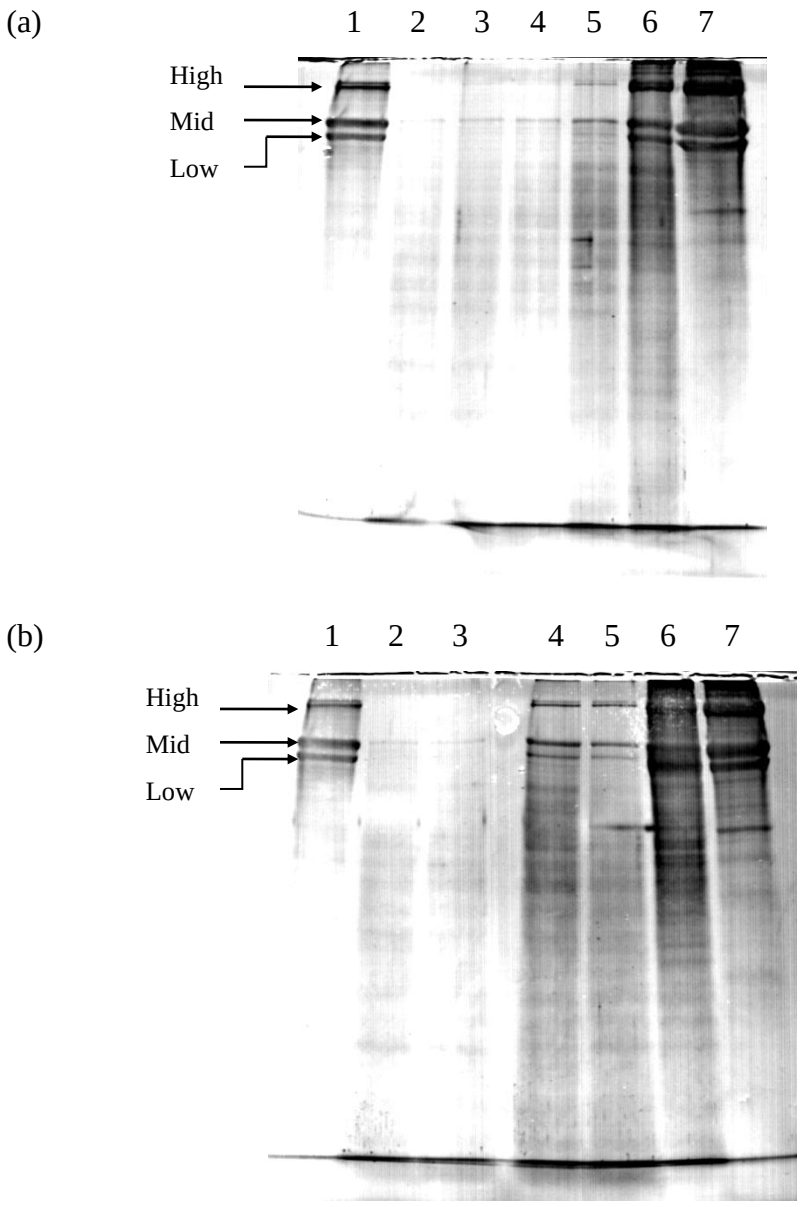


Figure 3.11. SDS-PAGE images from collagen degradation experiments using bacterial collagenase from *clostridium histolyticum*. For both images: lane 1 is collagen, lane 2 is collagen with 1 unit collagenase, lanes 3, 4, 5, 6 and 7 are collagen with 1 unit collagenase and 0.25, 1, 0.5, 2, and 5 mM ACE inhibitor respectively. (a) Captopril treated samples. (b) Enalapril treated samples.

The samples treated with enalapril also showed evidence of collagenase inhibition at lower concentrations, with the high, mid, and low bands measuring 50.8 ± 22.7%, 33.4 ± 16.3%, and 40.8 ± 14.5% of their respective controls at 0.5 mM enalapril compared with 11.8 ± 4.7%, 8.9 ± 1.3%, and 6.1 ± 1.1% for the bands from the samples treated only with collagenase *(Fig. 3.12(b))*. For the higher tested concentrations (2 mM and 5 mM) the bands again measured more than those of the controls, with the high, mid, and low bands measuring 232.9 ± 103.7%, 115.2 ± 39.1%, and 151.4 ± 51.2% of their respective controls at 5 mM enalapril *(Fig. 3.12(b))*. However, when compared with the samples treated with only collagenase the only statistically significant differences noted were for the mid and low bands at 5 mM enalapril ($p < 0.05$).

(a) Captopril

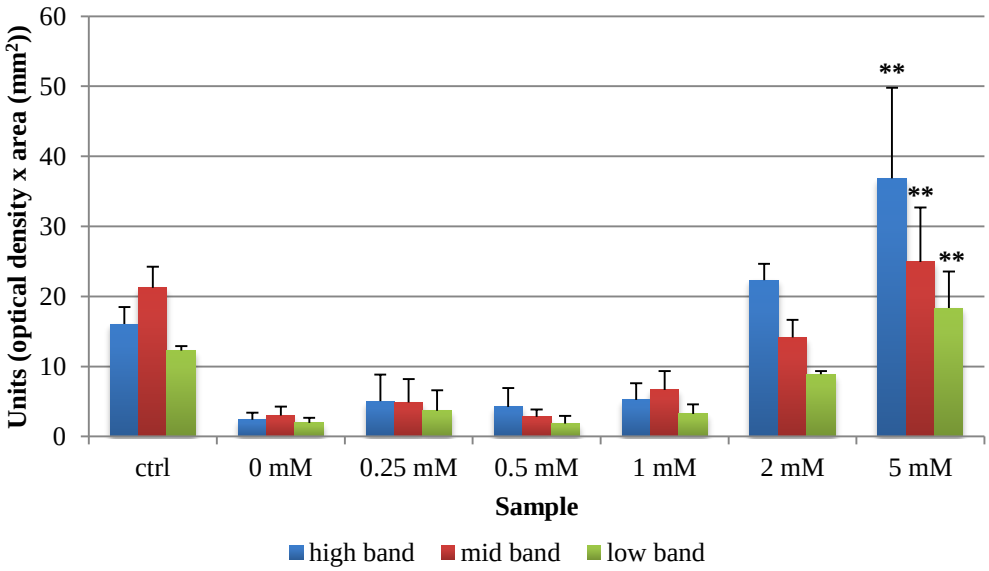
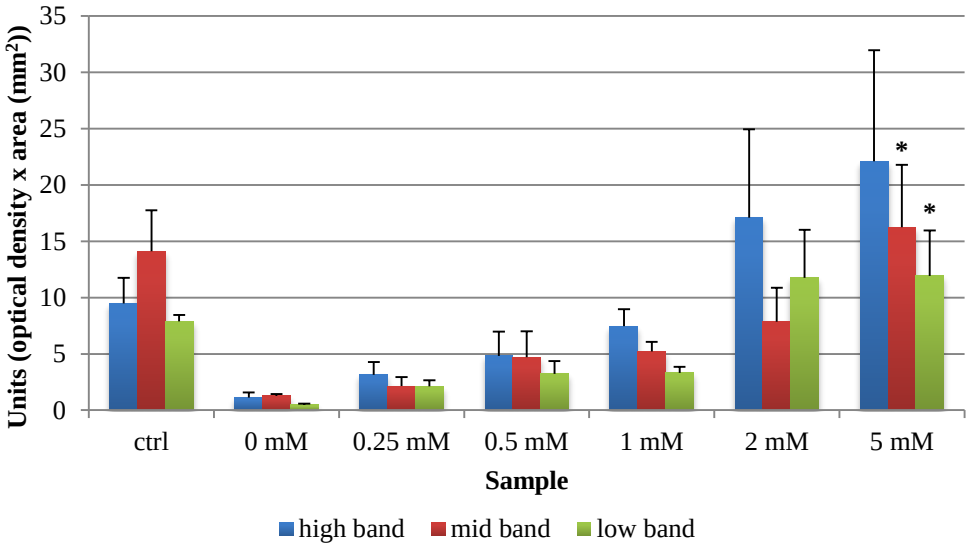


Figure 3.12. Analysis of SDS-PAGE images from experiments using bacterial collagenase from *clostridium histolyticum*. Bars represent relative optical densities of the three main collagen bands. (a) Analysis from captopril treated samples. (b) Analysis from enalapril treated samples. For both charts the control sample ('ctrl') was collagen only, while all other samples were collagen treated with 1 unit bacterial collagenase from *clostridium histolyticum* and concentrations of ACE inhibitors as detailed on the charts. Results are mean + SEM, n = 3. Results compared using ANOVA followed by Dunnett's test with respect to 0 mM treated samples. Significant variations: * $p < 0.05$, ** $p < 0.01$, 95% confidence.

(b) Enalapril



In view of the fact that SDS-PAGE experiments showed an amplified signal for collagen in the presence of higher concentrations of enalapril and captopril, further experiments were performed to examine whether the ACE inhibitors may be affecting the results in a manner other than through inhibition of degradative enzymes. It was shown that when 5 mM enalapril or captopril had been incubated with collagen (and no collagenase) there was an amplified signal of the three main collagen bands compared with collagen alone, and also evidence of many other protein bands that were not observed for the pure collagen sample including a more intense upper band representing the trimeric form of collagen (γ-band) *(Fig. 3.13(a))*. There was an absence of any signal where enalapril and captopril had been incubated without collagen or collagenase *(Fig. 3.13(b))*.

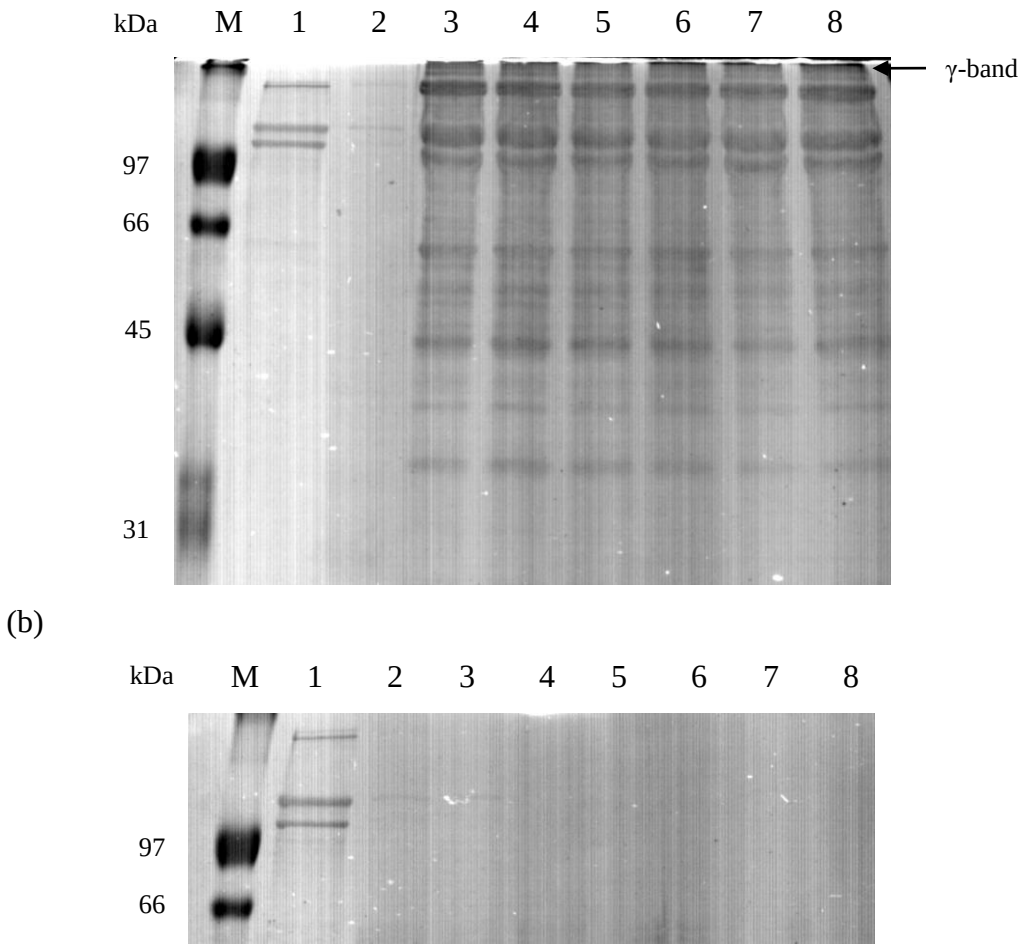


Figure 3.13. SDS-PAGE images from collagen degradation experiments using bacterial collagenase from *clostridium histolyticum*. For both images M is the molecular weight marker, lane 1 is collagen, and lane 2 is collagen with 1 unit collagenase. (a) Lanes 3, 4 and 5 are collagen with 5 mM enalapril and lanes 6, 7 and 8 are collagen with 5 mM captopril (no collagenase). (b) As (a) but with no collagen.

Analysis of the three main collagen bands from experiments without collagenase was compared with analysis from previous experiments with collagenase and similar concentrations of ACE inhibitors *(Fig. 3.14).* The collagen samples treated with collagenase and enalapril or captopril had average band intensities of 113.7 ± 43.7% and 180.9 ± 58.3% of control respectively, while the samples treated with only

enalapril or captopril had intensities of 150.0 ± 16.5% and 122.7 ± 12.0% of control respectively. Although there was a perceived increase in the band sizes and intensities relative to the collagen control for all ACE inhibitor treated samples, there were no statistically significant differences. The only significant differences (marked on *Fig.3.14*) were observed for certain of the ACE inhibitor treated samples when compared with the samples treated only with collagenase. There were no significant differences between the collagen samples treated with both ACE inhibitors and collagenase and the samples that had been treated with ACE inhibitors only. However, it should be noted that the collagen samples that had been treated only with ACE inhibitors were all from the same batch of collagen, while the results from samples treated with both ACE inhibitors and collagenase are from experiments where the same batch of collagen or collagenase was not necessarily always used.

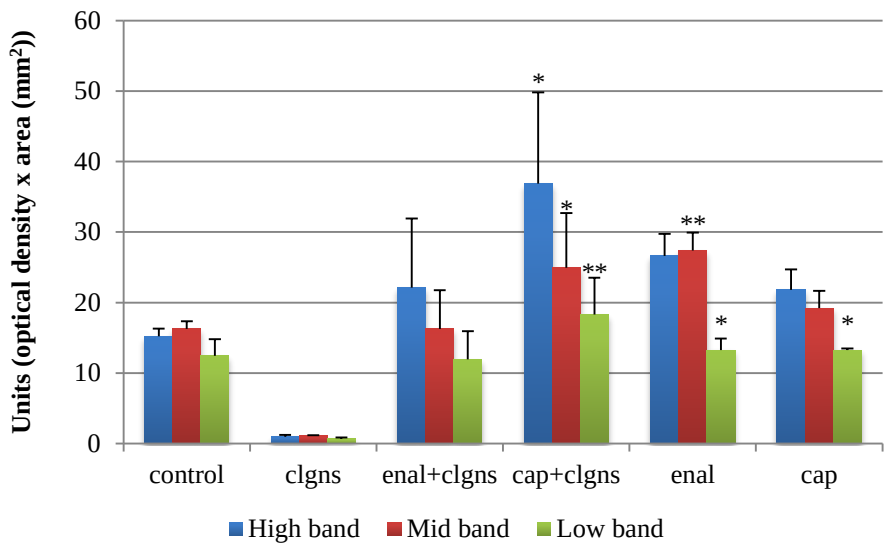


Figure 3.14. Comparative analysis of SDS-PAGE images from experiments in the presence or absence of bacterial collagenase from *clostridium histolyticum*. 'Control' is collagen, 'clgns' is collagen with 1 unit collagenase, 'enal+clgns' and 'cap+clgns' are collagen with 1 unit collagenase and 5 mM enalapril and captopril respectively, and 'enal' and 'cap' are collagen with 5 mM enalapril and captopril respectively (no collagenase). Bars represent relative optical densities of the three main collagen bands. Results are mean + SEM, n = 3. Results compared using ANOVA followed by Tukey's range test. Significant variations: * $p < 0.05$, ** $p < 0.01$, with respect to 'clgns', 95% confidence.

For the majority of the SDS-PAGE experiments, relatively large variations in the measured band intensities meant that statistically significant levels of collagenase inhibition were only achieved for the higher concentrations of ACE inhibitors tested. However, the results clearly suggest that both captopril and enalapril can inhibit the proteolytic degradation of collagen in a dose-dependent manner, with signs of collagenase inhibition at ACE inhibitor concentrations as low as 0.25 mM.

3.4 Discussion

A major aim of this research was to attempt to control the degradation rate of collagen-based scaffolds by inhibiting the ability of collagenolytic enzymes to degrade the substrate. It was therefore important to find experimental methods to assess the abilities of selected agents to inhibit digestion of collagen by collagenase.

The standard protocol for measuring collagen degradation via the colourimetric ninhydrin method of Moore & Stein (1948) was initially employed for measuring the extent of collagen digestion in the presence of collagenase and ACE inhibitors. Unfortunately, high background amino acid levels from the rat-tail collagen and inexplicable large variations in readings from samples led to inconclusive results, and alternative techniques to assess collagenolytic degradation and inhibition were sought.

3.4.1 Collagen Zymography

Gelatin and casein zymography have been successfully used for many years to analyse and quantify the ability of appropriate enzymes to degrade the substrates (Snoek-van Beurden & Von den Hoff, 2005). In fact, gelatin zymography has previously been used successfully to examine the ability of captopril to inhibit certain gelatinases (Sorbi *et al*, 1993), and it was therefore hypothesised that a similar methodology using collagen as a substrate would be suitable for analysing collagenase inhibition by ACE inhibitors. Although it was originally thought that, due to its complex structure, incorporating native collagen into polyacrylamide gels would be unsuitable for zymography, Gogly *et al* (1998) demonstrated that collagen zymography was not only possible, but was in fact a highly sensitive and quantifiable technique for the analysis of interstitial collagenase. However, collagen zymograms are also notorious for being technically difficult and are not widely used (Troeberg & Nagase, 2001). It was consequently decided that if preliminary experiments deemed the technique to be viable for detection of collagen digestion by bacterial

collagenase, it would be developed further to also test for inhibition of collagenase by enalapril and captopril.

3.4.1.1 Zymography: Validation

Initial zymography studies at room temperature suggested the technique to be sensitive to bacterial collagenase activity, and although areas of lysis were too large for analysis and had merged with each other, a shorter incubation time remedied this issue. However, the initial studies also gave rise to problems surrounding a large unstained area at the top of the zymograms that overlapped with the areas of enzymatic degradation and hence affected results. Since the Coomassie stain binds to proteins within the substrate, this upper unstained area suggests that the collagen within the gel is possibly being mobilised by the electrophoresis. Collagen in its triple-helical conformation should be relatively immobile, but denatured collagen is susceptible to electrophoretic mobility, and since the collagen would have been partially denatured by the SDS this movement should perhaps not be entirely unexpected. The exchange of SDS with Triton X-100 during the later stage of incubation is said to restore part of the original triple-helical conformation of collagen (to ensure its specificity for degradation by collagenases) (Snoek-van Beurden & Von den Hoff, 2005), but since this process happens after the electrophoresis stage it appears difficult to ensure that the collagen has not been previously denatured by the SDS to the point that it is able to migrate through the gel. This problem is apparently common during collagen zymography, and images of collagen zymograms with similar unstained areas that overlap the areas of enzymatic degradation have even been published in high impact journals (Goo *et al*, 2003). Nevertheless, steps were taken to try and alleviate this troublesome issue.

It was hypothesised that relatively high ambient temperatures during electrophoresis could be contributing to denaturation of the collagen, and a decision was made to try running gels at 4 °C instead of at room temperature. The result of these lower temperature runs was that the large unstained area no longer appeared, but also that only a single lysis band was evident for each lane of the zymogram instead of

multiple bands that had been present when the gels were run at room temperature. While the lysis band detected from zymograms run at 4 °C was clear and well defined, it was initially considered more useful to be able to detect multiple collagenolytic fragments if they were present. The ability to examine multiple lysis bands individually gives greater insight into the specific inhibitory properties of the drugs being tested, as it is then easier to ascertain whether the drugs are broad-ranging inhibitors or have a higher affinity for specific proteases. Zymograms run at approximately 15 °C did not vary in appearance from those run at 4 °C, suggesting that the temperature at which collagen would run into the gel was at least higher than this intermediate temperature.

Several further methods were tried to improve resolution at this lower temperature. Increasing the voltage for electrophoretic separation resulted in less well-defined bands than were present for lower voltage runs, but still only a single band per lane, suggesting that the applied voltage was not an overriding factor. Increasing the time for electrophoretic separation at 4 °C also appeared to have no effect on the number of lysis bands detectable. Altering the polyacrylamide content of the gels had an effect on resolution, but not on the number of detectable lysis bands. Lowering the polyacrylamide content resulted in lysis bands with an irregular shape and poor definition, while increasing it led to lysis bands that would barely migrate into the gel at all and were therefore extremely difficult to analyse. As 10% polyacrylamide gels appeared to give the best resolution these were used for all subsequent zymography experiments.

Effects of alterations to the pH of the polyacrylamide gels were also studied, and it was noted that lowering the pH of gels run at room temperature gave a far greater spread of lysis bands and much better definition, although the issue of the higher molecular weight fragments falling within an unstained upper region was still apparent. Raising the pH led to all bands overlapping and falling within this upper unstained region and so could only be considered disadvantageous. It is also worth noting that lowering the pH of a polyacrylamide gel to 8 brings it closer to physiological pH and so would be more suitable for the study of enzyme interactions with mammalian substrates, whereas raising the pH to 9 takes it further from the

optimal physiological range for collagenase activity (Mookhtiar & Van Wart, 1992). A combination of lowering the pH and running gels at 4 °C still resulted in only a single lysis band being observable, suggesting that the temperature of the electrophoresis was the dominant factor in achieving multiple lysis bands, while alterations to pH simply affected the spread and resolution of the bands.

Despite the prevalent issues with unstained upper regions of zymograms following electrophoresis, a decision was made that all subsequent zymograms would be set at the lower pH, run at 125 V for approximately 2.5 hours and at room temperature, and then incubated in zymogram developing buffer for 18 hours at 37 °C, as this combination of experimental conditions appeared to give the most varied and usable results.

It has since been realised that it may also in fact have been beneficial to run the zymograms with the ACE inhibitors at 4 °C. Although only a single lysis band was detected at 4 °C, this suggests that the collagen within the substrate may have retained more of its native structure, since only a pure collagenase would be expected to degrade triple-helical collagen.

The collagenase preparation used is a mixture containing not only specific collagenases, but also other less-specific proteases that are only capable of digesting collagen after it has already been partially denatured (see **section 3.1.1**). It is thought that the zymograms run at room temperature were more likely to have contained partially denatured collagen (i.e. gelatin), and therefore it is less certain whether the areas of enzymatic degradation are attributable to true collagenases or to other less specific proteases. The exchange of SDS with Triton X-100 is said to partially restore the triple-helical nature of collagen for zymography (Snoek-van Beurden & Von den Hoff, 2005), but without knowing the precise specificity of the degradative enzymes being tested it is difficult to know whether the substrate truly is native collagen, or a form of denatured collagen. The fact that the collagen did not appear to migrate through the gels at 4 °C suggests that the collagen within it had retained more of its original structure, which may also have made it susceptible to fewer of the proteolytic enzymes in the bacterial collagenase mixture. However, it is also possible that the full range of degradative enzymes within the collagenase mixture

were simply not able to migrate far enough into the resolving gel at 4 °C to be detectable.

Despite the lack of certainty concerning the specifics of the substrate and the degradative enzymes, the technique is still capable of quantifying the degradation of the substrate by bacterial collagenase. However, the results presented here also highlight some of the inherent difficulties of collagen zymography, which are perhaps partly responsible for the relative unpopularity of the technique.

3.4.1.2 Zymography: ACE Inhibitor Studies

At this stage in experimentation both a new batch of collagen and a more concentrated Coomassie stain were employed, and consequently zymograms no longer appeared as badly affected by unstained background regions in the upper section. It is not known whether this development is attributable to a single one of these alterations to the procedure or a combination of both. It did, however, make analysis of individual bands much more straightforward for all subsequent zymograms.

For zymograms incubated in the developing buffer in the absence of ACE inhibitors there were a total of five clearly visible lysis bands for the higher quantities of collagenase tested suggesting that the bacterial collagenase contained at least five distinct polypeptides capable of substrate digestion. However, as was stated in the previous section, it is unclear as to whether the substrate in fact contained collagen in its native triple-helical conformation, or collagen that had already been partially denatured. Since denatured collagen would be susceptible to other MMPs that are less-specific than true collagenases, it cannot be concluded with certainty that collagenase activity, and thus collagenase inhibition, were being detected during the zymography experiments. However, despite the uncertainty of knowing precisely the nature of the substrate and therefore the enzymes being detected, the experiments were clearly able to detect substrate degradation by MMPs.

For lower quantities of bacterial collagenase the two lower molecular weight fractions were not discernable, but the three upper molecular weight fractions still produced noticeable substrate lysis, allowing a relationship between the total quantity of bacterial collagenase and the amount of substrate lysis by individual constituent enzymes to be determined for these upper bands. All three of the upper molecular weight fractions confirmed strong relationships between collagenase quantity and substrate lysis, although the type of relationship varied. While the two highest molecular weight bands tested demonstrated a logarithmic relationship between collagenase quantity and substrate lysis, the lowest molecular weight band tested revealed a highly linear relationship, suggesting that the correlation between the quantity of a degradative enzyme and the amount of corresponding substrate lysis in zymography cannot be assumed to be similar for all proteolytic enzymes with an affinity for that substrate. However, these strong correlations between collagenase quantity and amount of substrate lysis validate the method as a sensitive and quantifiable technique for the determination and analysis of substrate degradation by non-specific bacterial collagenase.

EDTA was chosen as a positive control as it has been suggested to be a potent inhibitor of MMPs (Gogly *et al*, 1998). Indeed, zymograms incubated with 5 and 10 mM EDTA demonstrated no evidence of substrate lysis, confirming that the inhibition of enzymatic degradation was taking place. The zymograms incubated with enalapril also demonstrated marked reductions in the levels of substrate degradation; even at 5 mM enalapril there was only faint evidence of substrate lysis and only for the higher quantities of collagenase tested. However, there was little perceived difference at 10 mM enalapril, implying that there may be a limit to the amount of enalapril that can be effective in MMP inhibition. Densitometry analysis of the three highest molecular weight collagenase fragments for both 5 and 10 mM enalapril treated samples confirmed these findings, with over 90% inhibition being achieved with 5 ng ($p < 0.001$) and 10 ng ($p < 0.01$) collagenase.

Zymograms incubated with 5 mM captopril appeared similar to untreated zymograms, with only small signs of inhibition of substrate lysis, again suggesting that captopril is not as potent a MMP inhibitor as enalapril. However, zymograms

incubated with 10 mM captopril demonstrated much greater degradative inhibition, and the pattern of lysis was similar to that of the enalapril treated zymograms, confirming that captopril is an inhibitor of ECM-degrading enzymes and that its effects are dose dependent. Image analysis (densitometry) of the three highest molecular weight collagenase fragments demonstrated that 5 mM captopril was in fact sufficient to significantly inhibit substrate degradation by 5 ng collagenase ($p < 0.01$), and there was also marked inhibition of substrate degradation with 10 ng collagenase (approximately 60% inhibition) though this was not statistically significant. However, 10 mM captopril led to almost a complete disappearance of these upper bands for both 5 ng ($p < 0.001$) and 10 ng ($p < 0.01$ for the two upper bands, $p < 0.05$ for the lower band) collagenase, verifying that captopril inhibits substrate degradation by bacterial collagenase in a dose dependent manner.

It is of note that previous zymographic studies with ACE inhibitors suggest that required concentrations for inhibition of enzymatic degradation do not reflect the real inhibitory capacity of the agents tested as high concentrations are required to penetrate the polyacrylamide-substrate complex, stating that captopril concentrations of up to 40 mM are required for gelatinase inhibition during gelatin zymography (Sorbi *et al*, 1993). It is therefore likely that under different circumstances enalapril and captopril may be able to inhibit bacterial collagenase at much lower concentrations.

3.4.2 SDS-PAGE Analysis

SDS-PAGE has long been used as a method for protein analysis due to its relative simplicity and its ability to separate and characterise proteins regardless of their inherent solubility in aqueous solution (Chiou & Wu, 1999). The process of collagen degradation by collagenase involves the cleavage of large collagen monomers into smaller subunits, and it was hypothesised that SDS-PAGE may also allow characterisation of these smaller degradation products (in addition to the larger native collagen polypeptide chains), and consequently provide a qualitative assessment of the degree of collagen degradation that may be taking place.

3.4.2.1 SDS-PAGE Analysis of Collagen Degradation with Mammalian Collagenase

Using collagen solutions that had been isolated from rat tail tendon allowed for clear visualisation of the collagen monomers and their dimeric and trimeric forms where present. However, early experiments using the rat tail collagen in combination with mammalian cell homogenate were somewhat inconclusive. The addition of the cell homogenate did not appear to affect the presence or intensity of the main collagen bands, and it was extremely difficult to distinguish smaller polypeptide bands that could perhaps have been the result of degradation of larger collagen monomers from polypeptides inherent to the cell homogenate itself. It was also difficult to ascertain whether there were any differences in the amount of collagen degradation between samples where ACE inhibitors had been added at the time of assay, and samples where ACE inhibitors had been incubated with cells before they were homogenised. Although not confirmed, it is extremely likely that the cell homogenate samples would have contained specific collagenases. However, it is also likely that the activity of these enzymes was not great enough to sufficiently degrade the collagen substrate, or that they required some form of activation.

One particular batch of the 3T3 cell homogenate did achieve sufficient breakdown of the collagen molecules, though it is not understood why this batch appeared to induce more degradation than others that had been obtained in a similar manner. Experiments using this cell homogenate as a source of collagenase clearly showed that inhibition of collagenolytic degradation was taking place in samples that had been treated with ACE inhibitors at the time of assay. It was also clear that collagenase inhibition was taking place in a dose dependent manner as there were more intense bands representing the collagen molecules at higher concentrations of both enalapril and captopril, and also less evidence of smaller degradation products at these higher concentrations.

Overall, the results from experiments using mammalian cell homogenate were inconsistent and difficult to replicate. It had been assumed initially that simply preparing a cell homogenate from fibroblast monolayers would provide sufficient collagenase activity to induce collagen degradation *in vitro*. However, it is now

thought that isolation of specific collagenolytic proteins, and more importantly activation of latent pro-collagenases by the use of APMA (Murphy *et al*, 1977), would have resulted in increased substrate degradation and more conclusive results. Culturing the cells on collagen, as opposed to tissue culture plastic, may also have induced greater levels of activated MMPs to be produced. For all subsequent experiments only bacterial collagenase from *clostridium histolyticum* was used to degrade the collagen substrates where required.

3.4.2.2 SDS-PAGE Analysis of Collagen Degradation with Bacterial Collagenase

The use of bacterial collagenase allowed for much clearer visualisation of collagen breakdown by SDS-PAGE analysis. Preliminary experiments with this type of collagenase demonstrated that it was able to completely dissociate the collagen monomers, but there was little evidence of degradation products further down the gels. The absence of bands representing lower weight peptides is likely a consequence of the very broad substrate specificity of the bacterial collagenase (compared with mammalian collagenase), which is able to degrade not only the water-insoluble native collagens, but also further degrade water-soluble denatured ones (Mookhtiar & Van Wart, 1992). It is thought that the bacterial collagenase could be lysing the collagen into such small fragments they are undetectable within the resolution of the polyacrylamide gels used and simply run off the end of gel before the assay is complete.

These early experiments with bacterial collagenase suggested that one unit of collagenase was sufficient to completely degrade the native collagen, while 1 mM enalapril could successfully inhibit the degradative capability of up to 5 units of collagenase. Captopril also appeared to inhibit the action of collagenase, though not in as effective a manner as enalapril, with less intense protein bands (compared to control and enalapril treated samples) for incubations with 1 unit collagenase, and no sign of inhibition at 5 units collagenase. The results implied that enalapril was a more potent collagenase inhibitor.

For subsequent experiments collagenase content was kept constant at one unit where required, and varying amounts of enalapril and captopril were tested for collagenase inhibition in an effort to quantify the amount of inhibition achievable by each of the ACE inhibitors. While there was evidence of collagenase inhibition even at the lowest tested concentrations (0.25 mM) of both enalapril and captopril, these findings were not statistically significant, and there was also little evidence of much variation in the level of inhibition for 0.25, 0.5, or 1 mM concentrations of either drug. At 2 mM concentrations of each drug there was a marked (though still not significant) increase in the level of collagenase inhibition achieved. However, according to densitometry analysis of the images, at 5 mM concentrations of enalapril and captopril bands representing the main collagen monomers actually appeared amplified with respect to the collagen-only controls, and statistical significance was achieved for the mid and low bands from enalapril treated samples ($p < 0.05$) and all bands for captopril treated samples ($p < 0.01$) with respect to the collagen samples treated only with collagenase. It is quite clear from the SDS-PAGE images and the accompanying densitometry analysis that inhibition of collagen degradation was taking place even at the lower concentrations tested. The lack of statistical significance is attributable to large variations in the results of subsequent experiments, and it is thought that significance could have been achieved for lower concentrations if more experiments were carried out and with more stringent controls. Possible reasons for these large discrepancies in results could be differences between batches of collagen or collagenase, and variations in ambient temperature while performing SDS-PAGE.

In order to examine whether enalapril or captopril may be affecting the results in a manner other than through inhibition of collagenolytic enzymes, further experiments were performed incubating the drugs in the presence or absence of collagen and without collagenase. The absence of any signal where enalapril and captopril had been incubated without collagen or collagenase suggests that the mere presence of ACE inhibitors is not detectable by SDS-PAGE, and therefore that any unexpected effect on the results is more likely attributable to the interaction of the drugs with either the substrate or the enzymes being assayed. Indeed, where enalapril and captopril were incubated with collagen and no collagenase, the bands representing

the collagen molecules appeared larger and more intense than those of the control samples, suggesting that the presence of ACE inhibitors was somehow affecting the results. These findings were further confirmed by image analysis and densitometry of the main collagen bands, where the average band intensities from collagen samples that had been treated with enalapril or captopril (and without collagenase) were greater than those of the control samples containing only collagen (not statistically significant), and similar to those from samples that had been treated with similar doses of the ACE inhibitors *and* collagenase. It was also noted from these latter experiments that incubation of enalapril or captopril with collagen and *without* collagenase resulted in many more polypeptide fragments being detected than simply those attributable to the main collagen monomers.

The mechanism by which the ACE inhibitors affect the banding pattern of collagen is not yet fully understood, but it appears that the drugs may be binding to the collagen, and in doing so stabilising the structure, preventing access for the collagenolytic enzymes, or both. Stabilisation of the collagen triple-helix seems likely since there is an obvious upper band representing the trimeric (quaternary) form of collagen (~400 kDa) which was present for the ACE inhibitor treated samples, but not for the untreated collagen *(Fig. 3.6(a))*. It is also worth noting that the molecular weight of a protein would increase when small molecules bind to it, which could account for the enlarging and spreading of the main collagen bands observed for the ACE inhibitor treated samples.

The appearance of additional bands further down the SDS-PAGE gel might initially suggest the presence of lower molecular weight fragments arising as a consequence of interactions between the collagen and the ACE inhibitors. However, it seems unlikely that the ACE inhibitors could themselves be denaturing the collagen due to the ordinarily highly specific nature of collagen-cleaving enzymes. In fact, it is hypothesised that the presence of these additional bands is more likely attributable to ACE inhibitor mediated folding of the collagen molecules. Since SDS-PAGE separates proteins not only by their molecular weight, but also by their relative *size*, it may be that these additional bands are simply the collagen monomers folded in such a way that they do not appear where expected.

It is possible that the ACE inhibitor molecules are specifically recognised by some component of the collagen polypeptide chains and incorporated via non-bonding interactions. Captopril contains a sulphydryl group, and it was thought that captopril could be stabilising the collagen molecule by binding to components within it and allowing the formation of intrachain disulphide bonds. However, the gels were run under reducing conditions (heating to near boiling point in the presence of 2-mercaptopethanol) which would normally be expected to completely reduce any disulphide bonds. Furthermore, the results using enalapril are similar to those using captopril, and since enalapril does not contain a sulphydryl group it appears that the ACE inhibitors may be folding the collagen in some other way; perhaps through the creation and stabilisation of hydrogen bonds (which would normally be disrupted by the SDS). This hypothesis appears reasonable since an increase in the number of stabilised intrachain hydrogen bonds could account for increased levels of protein folding leading to a number of bands appearing below the collagen monomers, while the stabilisation of existing interchain hydrogen bonds may explain the presence of bands representing the quaternary form of collagen which were not observed where captopril or enalapril were absent from the incubations.

Palama *et al* (2011) demonstrated that certain fluorophores can bind to collagen during its *in vitro* formation, and they hypothesised that the fluorophores binding to the collagen were most likely being recognised specifically by hydroxyproline and incorporated via non-bonding interactions within the polypeptide chain. It is thought that the ACE inhibitors in the present study may be affecting the stability of the molecules by binding to collagen in a similar manner, since hydroxyproline is said to play a major role in imparting structural stability to collagen via stereoelectronic effects and the formation of intra- and intermolecular bridges (Shoulders & Raines, 2009). The hydroxyproline is very likely to interact with the ACE inhibitors by hydrogen bonding and can potentially do so in a concerted fashion, the latter having a number of hydrogen-bond acceptor and donor groups that could facilitate bridging between different hydroxyproline residiues along or across the strands. However, further work is required to elicit the true nature of the ACE inhibitor interactions with the collagen substrate.

3.4.3 Conclusions from Collagen Degradation Studies

Although the ninhydrin colour development assay provided no useful results concerning the inhibition of collagenase by enalapril or captopril, other methods employed for the analysis of samples did give some indication of collagen digestion by collagenase and corresponding inhibition by enalapril and captopril. Having first validated its efficacy as a sensitive and quantifiable technique, zymography studies clearly demonstrated that both drugs were able to inhibit degradation of the substrate by the proteolytic enzymes within the bacterial collagenase. Although the zymograms probably contained triple-helical collagen, this cannot be confirmed, and it is possible that the collagen within the polyacrylamide gels was already partially denatured. Since the bacterial collagenase used contains a mixture of ECM-degrading enzymes, it is not clear from the zymography results whether the drugs are truly inhibitors of collagenase or were simply inhibiting the degradative action of less specific proteases. Despite these uncertainties concerning the purity of the collagen substrate, zymography studies did demonstrate that both drugs could inhibit all of the detected proteolytic fractions of bacterial collagenase in a dose dependent manner. It was initially hypothesised that captopril would be the more potent collagenase inhibitor as literature suggests that lisinopril (a drug very similar in structure to enalaprilat) has a similar inhibitory effect on gelatinase to captopril but at 1000 fold higher concentrations (Sorbi *et al*, 1993). It is therefore interesting to note that these experiments suggest enalapril to be the more potent inhibitor of the two.

Results from SDS-PAGE suggest that enalapril and captopril are in fact capable of inhibiting the degradation of collagen by collagenase. Despite problems achieving sufficient levels of collagen degradation when using cell homogenate as a source of mammalian collagenase, there was some indication of dose-dependent collagenase inhibition from both enalapril and captopril, though results were difficult to replicate. Using bacterial collagenase to degrade the collagen proved to be much more successful. While there was clear evidence of collagenase inhibition at concentrations of both enalapril and captopril as low as 0.25 mM, irregularities in results meant that statistically significant levels of inhibition were only achieved at the highest ACE inhibitor concentrations tested. It is supposed that these

irregularities may have resulted from variation between different batches of collagen or collagenase, and it is likely that more stringent controls would have resulted in significance being achieved at lower concentrations. Even so, the results from the SDS-PAGE studies using bacterial collagenase clearly show that both captopril and enalapril are able to inhibit collagenase-mediated collagen degradation in a dose-dependent manner, and that enalapril is the more potent inhibitor. However, the results also raised further questions about the mechanism (or mechanisms) of collagenase inhibition.

It was originally thought that the most probable mode of action for the inhibition of collagen degradation by ACE inhibitors is via chelation of Zn^{2+} at the active site (the S1' site) of MMPs, as has been suggested by Sorbi *et al* (1993) with respect to gelatinase inhibition. This hypothesis is compatible with the knowledge that other metal chelators such as EDTA, which was used as a positive control for the zymography experiments in this chapter, are potent MMP inhibitors. It has also been suggested that functional groups within larger ACE inhibitors may interact preferentially with the S1 site as opposed to the S1' site, effectively being stabilised by hydrophobic interactions and specific hydrogen bonds (Yamamoto *et al*, 2007a; Yamamoto *et al*, 2007b), although due to the small size of captopril such hydrophobic interactions are said to appear unnecessary for binding (Yamamoto *et al*, 2008).

While these previous studies have established that certain ACE inhibitors probably do bind to the active sites of MMPs and thus inhibit their proteolytic activity, some of the data acquired in the present study suggest that there may also be another mechanism involved in the inhibition of collagen degradation by enalapril and captopril. SDS-PAGE analysis of collagen that had been incubated with enalapril or captopril but without any degradative enzymes clearly demonstrated that the drugs were interacting with the collagen substrate, suggesting that their capacity for controlling collagen degradation may not be entirely attributable to the inhibition of degradative enzymes, but could also be due to stabilisation of non-covalent bonds within the collagen molecules. It is known that before collagen is able to hydrolyse peptide bonds it must first unwind the triple-helix, although the mechanism by which

it does this is not yet fully understood (Chung *et al*, 2004; Nagase & Fushimi, 2008). Therefore, it was hypothesised during the present study that enalapril and captopril may actually be binding to the collagen and interacting with it to strengthen existing bonds or to create new ones, preserving the native collagen's tightly bound structure and blocking access for degradative enzymes to unwind the triple-helix.

These new developments surrounding the proposed stabilisation of collagen triple-helices by ACE inhibitors, resulting in a reduction in their susceptibility to proteolytic degradation, could affect our overall understanding of the mechanisms by which some MMP inhibitors function. It appears that certain inhibitors of proteolytic degradation may in fact elicit their effect via an affinity for the substrate, rather than by binding to the MMPs themselves as is commonly proposed (Sorbi *et al*, 1993; Yamamoto *et al*, 2007a; Yamamoto *et al*, 2007b; Yamamoto *et al*, 2008).

Chapter 4

Cytotoxicity Studies

4 Cytotoxicity Studies

4.1 Introduction

4.1.1 Toxicology of ACE Inhibitors

ACE inhibitors have been successfully used in the treatment of hypertension for many years (Ferreira, 2000), and consequently much is known already about their safety and efficacy for purpose. There are a number of known adverse effects when the drugs are used therapeutically, and these can be separated into those that are specific to the entire class and those that are related to chemical structure (i.e. the presence of a sulfhydryl group). Potential side effects common to all ACE inhibitors include hypotension, hyperkalemia, cough, and dysgeusia (Brown & Vaughan, 1998). Furthermore, sulfhydryl-containing ACE inhibitors (e.g. captopril) have been known to cause neutropenia, nephrotic syndrome, and skin rash (DiBianco, 1986). However, none of the known side effects appear to imply anything about possible localised effects on cells in tissues. There is also considerable information available on the pharmacokinetic profiles of both captopril and enalapril when administered therapeutically for the treatment of hypertension (Al-Furaih *et al*, 1991; Biollaz *et al*, 1982; Johnston *et al*, 1984), with such studies focussing on blood serum concentrations of the drugs and their metabolites following oral (sublingual) administration. Yet there is scarce evidence of research into the potential toxicological effects of high doses of these agents, or to their effect at a cellular level. Since this particular body of research is concerned with completely novel uses for these therapeutic agents further studies into toxicological effects of the drugs, specifically with respect to cultured cells, were considered necessary.

One of the major aims of the present study was the incorporation of ACE inhibitors into tissue constructs designed to interact with cells, and it was therefore important to assess the effect of the presence of these drugs on cells within a matrix. It was of particular importance to investigate the effect of the drugs at the concentrations necessary for the inhibition of collagenase. The collagen degradation experiments (see **chapter 3**) suggest that these concentrations are considerably higher than would be expected in the serum of a patient following therapeutic administration of the

drugs, with reported blood serum levels unlikely to exceed 100 nM for enalapril (Biollaz *et al*, 1982; Johnston *et al*, 1984) or 1100 nM for captopril (Al-Furaih *et al*, 1991).

4.1.2 Cell Viability Studies

Fibroblasts were chosen for all cell studies as they are the most abundant cells in the connective tissue of mammals, and this research is largely concerned with the properties of collagen (the most abundant structural protein in the ECM) as a biomaterial and its interaction with cells. Furthermore, fibroblasts are associated with the synthesis of structural ECM proteins, as well as the natural enzymes (such as collagenase) and processes responsible for matrix degradation. Fibroblasts also play a critical role in wound healing and are known to actively remodel tissues both *in vivo* and *in vitro* (Grinnell, 1994). The 3T3 cells used in this research are immortalised mouse fibroblasts that were originally obtained from Swiss mouse embryo tissue (Todaro & Green, 1963), and have since become the standard fibroblast cell line for use with *in vitro* studies.

4.1.2.1 Two-Dimensional (2D) and Three-Dimensional (3D) Cell Studies

Historically the majority of cell studies undertaken using 3T3 cells have focussed on 2D culture techniques where single cell populations are cultured on flat, rigid surfaces such as tissue culture polystyrene, and consequently much of the testing within this chapter also focuses on the toxicity of captopril and enalapril to cells cultured in this way. However, it cannot be assumed that cell scaffolds are purely passive vehicles to allow the study of cell functions and gene expression, and it has been shown that the behaviour of cells *in vitro* can vary considerably when cultured on 3D matrices compared with the standard 2D approach (Petersen *et al*, 1992). It has been suggested that the ability of cells to migrate within the ECM and to remodel it depends as much on the physical and biochemical characteristics of a matrix as on the properties of the cells within it (Even-Ram & Yamada, 2005). It was therefore

considered important to also study the effect of the ACE inhibitors to cells cultured on collagen hydrogels, since they are more similar in structure to the natural ECM. The ECM itself can be considered a bioactive hydrogel scaffold that directs cell adhesion, proliferation, differentiation, morphology and gene expression while also providing mechanical support (Tibbitt & Anseth, 2009). 3D substrates are particularly useful when studying fibroblasts as these cells are not restricted by a polarising attachment to a basal lamina on one side.

4.1.2.2 MTT Assay for Mammalian Cell Viability

The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a colourimetric assay that reduces MTT (yellow) to formazan (purple) in the mitochondria and cytosol of living cells and as such can be considered a measure of the metabolic activity of living cells, rather than simply a measure of their viability (Mosmann, 1983).

4.1.2.3 Neutral Red (NR) Assay for Mammalian Cell Viability

The NR assay is another colourimetric assay which depends on the ability of the vital dye Neutral Red to bind to lysosomes within living cells (Borenfreund & Puerner, 1985). The strength of colour achieved allows for quantitative estimation of the number of viable cells present within a sample. Unlike the MTT assay, the response of this viability indicator is independent of metabolic activity or redox cofactor levels in the cells.

4.1.2.4 Epifluorescence Microscopy

Fluorescence microscopy is a form of microscopy where specimens are illuminated with light of a specific wavelength. The light is absorbed by fluorophores within the specimen, causing them to emit light of longer wavelengths (and thus different

colours) to the absorbed light. In epifluorescence microscopy the excitatory light is passed through the objective lens directly onto the specimen, instead of being transmitted through the specimen from below as for traditional light microscopy. The fluorescence in the specimen gives rise to emitted light of a different wavelength. It is only this emitted light along with reflected excitatory light that reaches the objective lens, since most of the excitatory light is transmitted through the specimen. A filter block containing excitation and emission filters can then be used to filter out any remaining excitatory light that would otherwise be visible through the objective lens.

For the purpose of this research epifluorescence microscopy was used in conjunction with biological fluorescent stains that bind to molecules of interest or specific cellular structures in order to view cell shape and morphology, in addition to ascertaining cell viability and functionality.

4.1.2.5 Live/dead Staining of Cell Monolayers with Ethidium Bromide (EtBr) and 5-Carboxyfluorescein Diacetate (CFDA)

EtBr cannot easily penetrate the membranes of living cells, and is therefore used as a stain for dead cells or cells in the final stages of apoptosis (Edidin, 1970). EtBr is an example of an intercalating stain, meaning that it is inserted between base pairs of DNA. It has an excitation wavelength of 545 nm and an emission wavelength of 605 nm (red).

EtBr is often used in conjunction with the living stain CFDA, for which samples are initially stained with fluorescein diacetate which is non-fluorescent and readily permeates cell membranes (Celada & Rotman, 1967). Having entered the cell the lipophilic blocking groups are cleaved by non-specific esterases, resulting in the fluorescent form carboxyfluorescein (CF) which only leaks slowly from cells (Persidsky & Baillie, 1977). CF has an excitation wavelength of 495 nm and an emission wavelength of 520 nm (green).

4.1.2.6 Live/dead Staining of Cell Monolayers with Acridine Orange (AO) and Propidium Iodide (PI)

Like EtBr, AO is an intercalating stain that is inserted between base pairs of DNA (Foglieni *et al*, 2001). However, AO is membrane permeable meaning that it stains cells whether they are living, dying or dead. Therefore, in order to ascertain cell viability, it must be used in combination with a stain that is membrane impermeable (such as PI). AO has an excitation wavelength of 490 nm and emission wavelengths of 530 nm and 640 nm, meaning that it can fluoresce both green and red.

PI is another intercalating stain that is membrane impermeable, binding to both DNA and RNA in dead or dying cells (Foglieni *et al*, 2001). It has an excitation wavelength of 530 nm and an emission wavelength of 615 nm (red).

4.1.2.7 Staining of Fibroblast-Populated Collagen Lattices (FPCLs) with Phalloidin-FITC and PI

Phalloidin is a phallotoxin that binds at the interface between F-actin subunits, locking them together and preventing their depolymerisation (Wulf *et al*, 1979). Consequently, by labelling phalloidin with fluorescent analogues it can be used to stain actin filaments for fluorescence microscopy and therefore to investigate the distribution of actin in cells and their surrounding matrix. The phalloidin used in this research was conjugated with fluorescein isothiocyanate (FITC) which has an excitation wavelength of 495 nm and an emission wavelength of 520 nm (green).

As phalloidin-FITC only stains actin it must be used in combination with another cell-specific stain in order to view actual cells within a sample. Cells are fixed in formalin before staining with phalloidin-FITC, which effectively kills the cells while still maintaining their shape and structure, allowing for the use of the membrane impermeable PI to stain the cell nuclei red.

4.1.3 Contraction of FPCLs

Floating fibroblast-populated collagen hydrogels (FPCLs) have long been used for the production of tissue-like structures, and are generally accepted as an appropriate model of wound contraction (Bell *et al*, 1979; Grinnell, 1994). Consequently, it was decided that FPCLs should be studied in the presence of enalapril and captopril to ascertain whether the ACE inhibitors would have any effect on the ability of the fibroblasts to contract the collagen substrate. Any differences in the amount of gel contraction between samples can most likely be attributed to differences in the function and viability of cells within the matrix.

4.1.3.1 Pulsed Ultra-Violet (PUV) Light Treatment

PUV light treatment was used for the purpose of inactivating cells, halting their proliferation, and therefore inhibiting their ability to contract the collagen substrate. This method of cell inactivation has already demonstrated strong biocidal effects for use in variety of applications, and does not appear to damage or alter the cell substrate in any way (MacGregor *et al*, 2008). PUV was used as a positive control for a toxic response in terms of inhibiting cell-mediated contraction.

4.2 Materials and Methods

4.2.1 Materials

All chemicals obtained from Sigma-Aldrich, Gillingham, Dorset, UK. Collagen prepared in-house from rat tail tendons according to the method of Elsdale and Bard (1972), as described in **section 2.1.1**.

4.2.2 Measurement of cell viability: MTT and NR assays

For both assays 3T3 cells were seeded in 96-well plates at a concentration of $2x10^4/cm^2$ in growth medium and incubated overnight at 37 °C (5% CO_2 in air) to allow the cells to attach to the base of the wells. Following this, the medium was removed and either replaced with fresh medium or with medium containing the dissolved drugs at appropriate concentrations (sterile filtered). Both captopril and enalapril act to make the medium slightly more acidic, so pH was readjusted with drop wise addition of 0.5 M NaOH prior to sterile filtration. Plates were then incubated at 37 °C for a further 72 hours, before carrying out the MTT or NR assays.

4.2.2.1 MTT assay

10 mM solution of MTT in PBS pH 6.75 was made up and filtered through a 0.2 µm filter. Once the incubation period was complete the medium was removed from the 96-well plate and 50 µl MTT solution was added to each well. The plate was then incubated for a further 4 hours at 37 °C before removing the MTT solution. 200 µl dimethylsulfoxide (DMSO) was then added to each well to dissolve the formazan product and was mixed to an even colour. Absorbance was read at 540 nm using a BIO-RAD (Model 450) microplate reader. Results were compared by ANOVA followed by Dunnett's test.

4.2.2.2 NR assay

5 mg NR was dissolved in 100 ml PBS and incubated overnight at 37 °C before passing it through a 0.2 µm filter. Destain was made from ethanol, glacial acetic acid, and dH_2O at a ratio of 50:1:49. Once the 72 hour incubation period was complete the medium was removed from the 96-well plate and 100 µl NR solution was added to each well. The plate was then incubated for a further 3 hours at 37 °C. NR solution was then removed and the cells were washed once with PBS (200 µl/well). At this stage 100 µl destain was added to each well and the plate shaken until a homogenous

colour was achieved in all wells. Again the absorbance was read at 540 nm using the microplate reader. Results were compared by ANOVA followed by Dunnett's test.

4.2.3 Epifluorescence microscopy

For monolayer studies 3T3 cells were seeded in 60 mm Petri dishes in 5 ml growth medium at a concentration of $1x10^3/cm^2$ and incubated at 37 °C overnight to allow the cells to adhere, before the growth medium was removed and replaced either with fresh medium or with medium containing dissolved 1 mM captopril or enalapril. Samples were then incubated for 72 hours at 37 °C.

4.2.3.1 Live/dead Staining of Cell Monolayers with EtBr and CFDA

CFDA (Sigma, C4916) was prepared at 25 µM in PBS pH 6.75 from a stock solution of 2.5 mM in DMSO. EtBr was prepared at 100 µg/ml in PBS pH 6.75.

Growth medium was first removed from the Petri dishes and samples were washed two times with PBS. 2 ml EtBr was then added to each sample and they were incubated in the dark for 6 min at room temperature. The EtBr was removed and the samples washed three times with PBS. 3 ml of CFDA was then added to each sample and they were incubated at 4 °C for 5 min. CFDA was then removed and the samples were rapidly given a further two washes with PBS. Samples were then viewed in PBS with a ×20 (0.5 NA) water lens using a Zeiss AxioImager Z1 wide field fluorescence microscope and digital imaging system.

4.2.3.2 Live/dead Staining of Cell Monolayers with AO and PI

AO and PI were prepared at 20 µg/ml and 100 µg/ml respectively in PBS pH 7.4.

The medium was first removed from the Petri dishes and the samples washed three times with PBS. 3 ml PI was then added to samples and they were incubated in the

dark for 1 min at RT. The PI was removed and the samples were washed again three times with PBS. 3 ml AO was then added and samples were incubated for a further 1 min at RT. Samples were given a further three washes with PBS before viewing in PBS with a ×20 (0.5 NA) water lens using a Zeiss AxioImager Z1 wide field fluorescence microscope and digital imaging system.

4.2.3.3 Staining of FPCLs with Phalloidin-FITC and PI

Stock solution of phalloidin-FITC was prepared in methanol at a concentration of 0.1 mg/ml and stored at -20 °C in the dark. The stock solution was diluted a further 1:10 in PBS (pH 7.4) before use.

0.3% w/v collagen gels were prepared in 35 mm Petri dishes according to the method of Elsdale and Bard (1972) at a volume of 1 ml/gel in order to minimise gel thickness and, in turn, aid light transmission for microscopy. As required certain collagen gels were prepared with 1 mM of either enalapril or captopril dissolved in their pre-gelled solutions.

3T3 cells were seeded onto the gels in 1.5 ml growth medium at a concentration of $1x10^4/cm^2$ and incubated at 37 °C overnight to allow the cells to adhere. Gels were then detached from the sides of the Petri dishes to allow them to float freely and incubated for a further 72 hours at 37 °C.

The medium was removed from the Petri dishes and the samples washed three times with PBS (pH 7.4). To fix the cells 4% formalin in PBS was added to the samples for a minimum of 24 hours (samples fixed in formalin could be stored indefinitely). Samples were then washed a further three times in PBS. 500 µl of the phalloidin-FITC in PBS solution was added to the top of each of the collagen gels and the samples were incubated at room temperature for 1 hour in a dark, moist chamber. Samples were again washed three times in PBS, with each wash lasting a minimum of 5 min. 1 ml PI was then added to samples that required nuclear staining for 1 min at room temperature before they were given a further three washes with PBS. Glass cover slips were carefully applied to the top surfaces of the gels and samples were

viewed with a ×20 (0.5 NA) dry lens using the Zeiss AxioImager Z1 wide field fluorescence microscope and digital imaging system.

4.2.4 Contraction of FPCLs

5 ml volume 0.3% w/v collagen gels were prepared in 60 mm Petri dishes according to the method of Elsdale and Bard (1972), with some gels containing captopril or enalapril at 1 mM as required. Cells were then seeded onto the gels at a concentration of $1x10^4/cm^2$ and incubated at 37 °C overnight to allow the cells to adhere. Gels were then detached from the Petri dishes and transferred into larger 100 mm Petri dishes and floated in 10 ml growth medium. The FPCLs were each photographed on top of standard graph paper and then incubated at 37 °C. The FPCLs were removed from the incubator and imaged in this way at approximately the same time each day for a further nine days, with growth media being replaced every three days. The resulting photographs were then analysed using the ImageJ (National Institutes of Health, *http://rsbweb.nih.gov/ij/*) software package to calculate the areas of the gels at the various time points. Results were then compared by two-way ANOVA followed by Dunnett's test with respect to both negative and positive controls.

4.2.4.1 PUV Light Treatment for FPCL Contraction Positive Control

For the purpose of killing cells, and hence halting cell proliferation, selected FPCLs were treated with ten single shot UV pulses of 1 kV shortly after being transferred to the larger Petri dishes. Equipment used was manufactured in-house by Samtech Ltd., UK, and consists of a 1 kV, capacitive discharge circuit which is used to drive a low pressure (450 torr), Xenon-filled flashlamp. The emission spectrum of the flashlamp contains a high UV content ideal for cell inactivation (MacGregor *et al*, 2008).

4.3 Results

4.3.1 Cell Viability Studies

4.3.1.1 NR assay

The NR assay showed that increasing concentrations of both drugs in growth medium resulted in varying effects on cell viability *(Fig. 4.1 (a, b))*. Although the lowest concentration of enalapril tested indicated a slight reduction in cell viability, at 10 and 100 µM concentrations there was a slight increase in absorbance to a maximum of 104.6 ± 2.0% of control, before falling to 85.2 ± 2.2% at 1 mM *(Fig. 1 (a))*. Results obtained for captopril showed a similar trend *(Fig. 4.1 (b))*, with a slight reduction in viability at 1 µM, but then a maximum absorbance of 106.1 ± 2.5% of control at 10 µM. Viability then dropped to a minimum of 87.0 ± 2.6% at 1 mM.

While there appears to be some variation in viability for the lower concentrations tested, the only significant differences when compared with the control samples were observed at the 1 mM concentrations for both enalapril ($p < 0.001$) and captopril ($p = 0.001$).

4.3.1.2 MTT assay

Though not significant, MTT assay suggested a slight increase in activity for the lowest tested concentration of enalapril, with 104.2 ± 2.3% of control at 1 µM, and almost no difference in activity at 10 and 100 µM *(Fig. 4.1 (c))*. At 1 mM enalapril there was a significant reduction in activity to 80.5 ± 2.2% ($p < 0.001$). MTT with captopril at 1 and 10 µM showed non-significant increases in activity of 106.6 ± 1.7 and 106.3 ± 1.8% of control respectively, while at 100 µM activity did increase significantly ($p < 0.001$) to 118.4 ± 1.6% of control *(Fig. 4.1 (d))*. At 1 mM captopril activity fell to 88.0 ± 3.2% ($p = 0.002$).

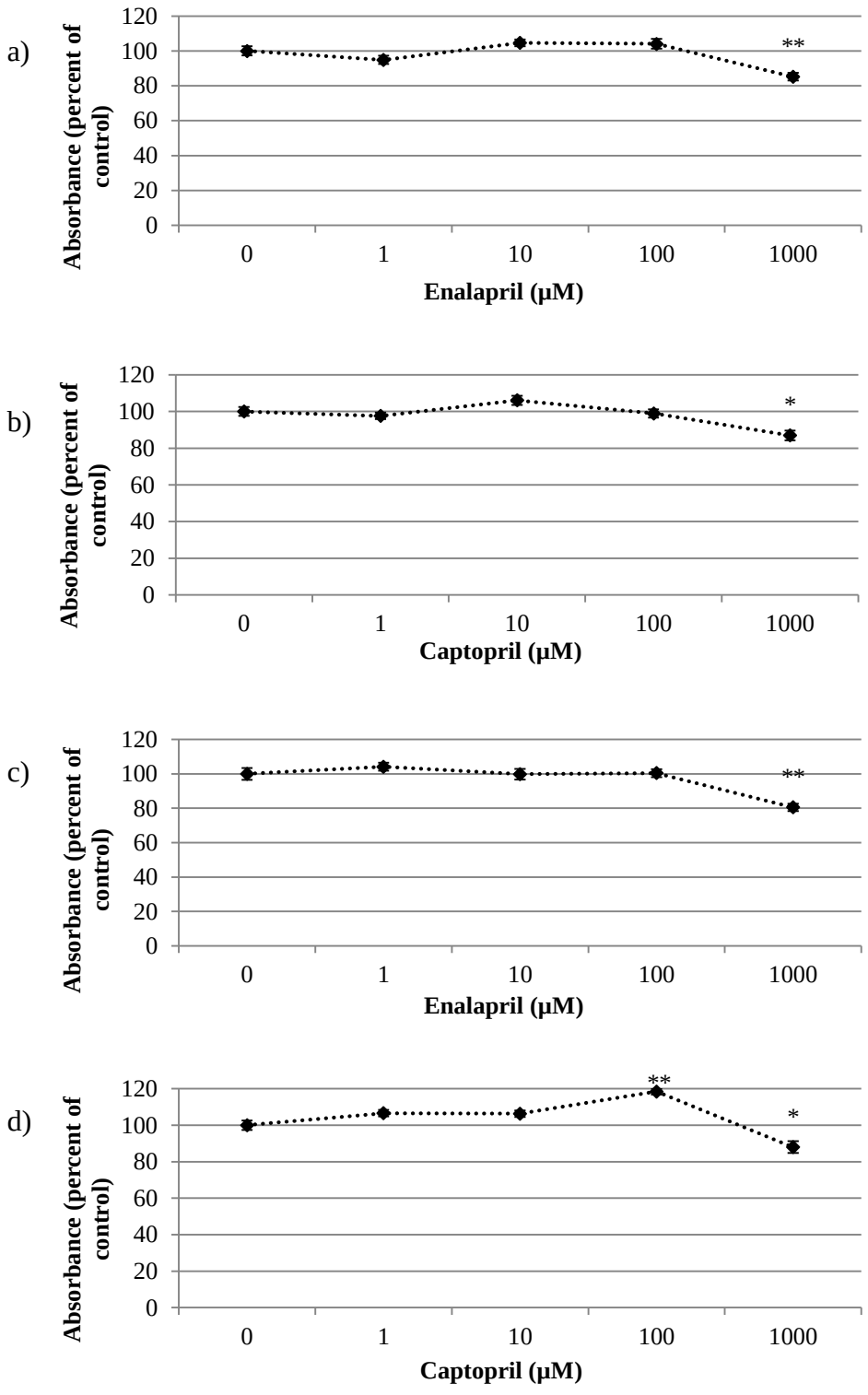


Figure 4.1. Viability of 3T3 cells ascertained by NR assay with enalapril (a) and captopril (b), and by MTT assay with enalapril (c) and captopril (d). Results are mean ± SEM, n = 12. Significant variations: * $P < 0.005$, ** $P < 0.001$

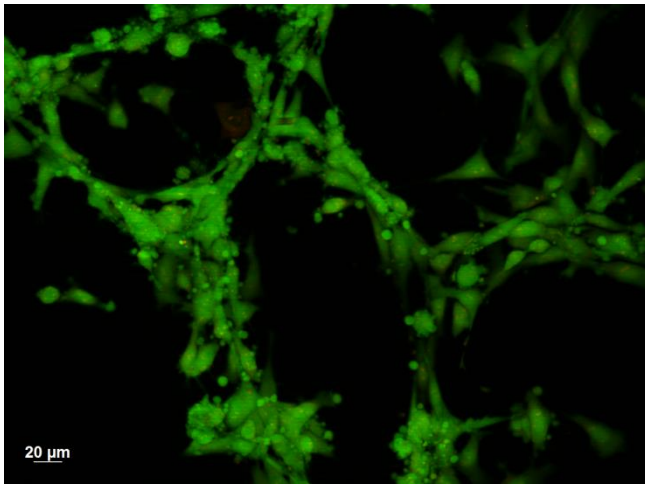
4.3.1.3 Live/dead Staining of Cell Monolayers with EtBr and CFDA

CFDA and EtBr were used initially for cell staining. While images of both untreated and ACE inhibitor treated cell monolayers displayed a high level of green fluorescence, indicating that the cells were viable, the use of these stains also resulted in increased levels of cell blebbing and apoptosis in all samples including negative controls *(Fig. 4.2)*. It was assumed that the abundance of apoptotic cells was most likely attributable to problems with the particular method or with the stains, and consequently other equally appropriate stains were chosen for cell staining and CFDA and EtBr were not used for any further experimentation.

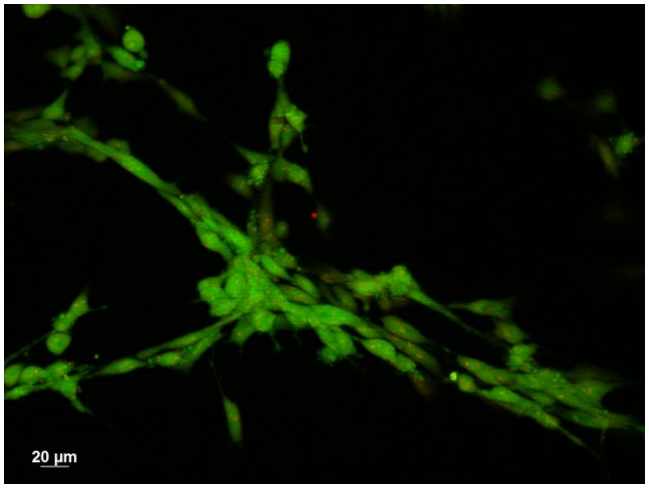
4.3.1.4 Live/dead Staining of Cell Monolayers with AO and PI

PI and AO were chosen for use as cell stains because, unlike CFDA and EtBr, these agents did not appear to have any toxic effect to the cells. Epifluorescent microscopy images suggest that cells retained high levels of viability and were generally healthy after 72 hours in culture both in the presence or absence of enalapril or captopril in growth medium *(Fig. 4.3)*. A green colour indicates healthy, living cells, and all three samples tested showed high levels of green fluorescence. A red colour indicates dead or dying cells, and although all images contained small amounts of red fluorescence, there did not appear to be a marked difference between the control and the ACE inhibitor treated samples. There was little evidence of cell blebbing or apoptosis from any of the samples.

a)



b)



c)

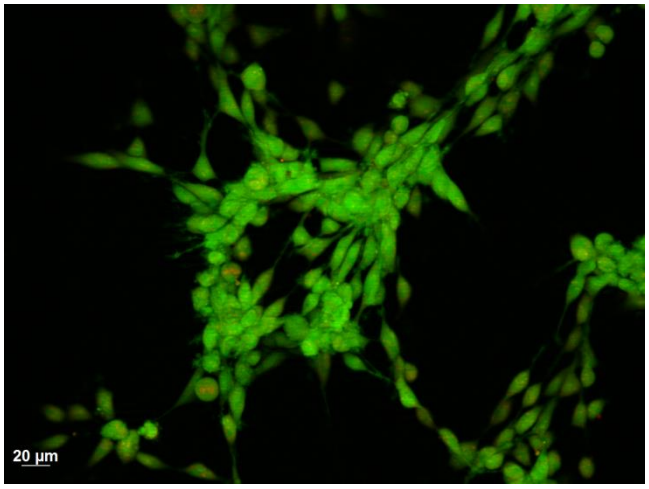
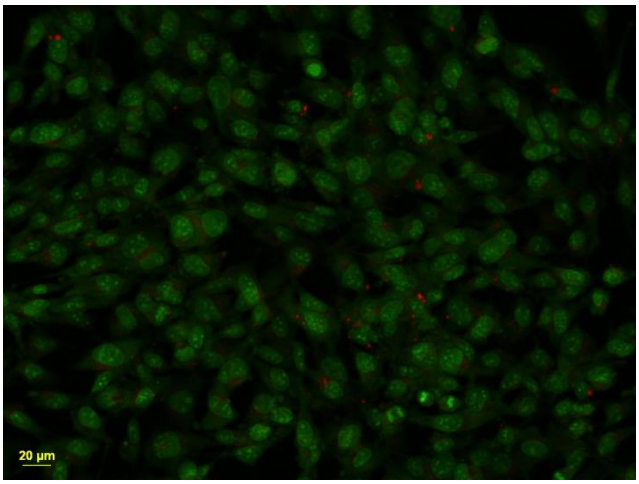
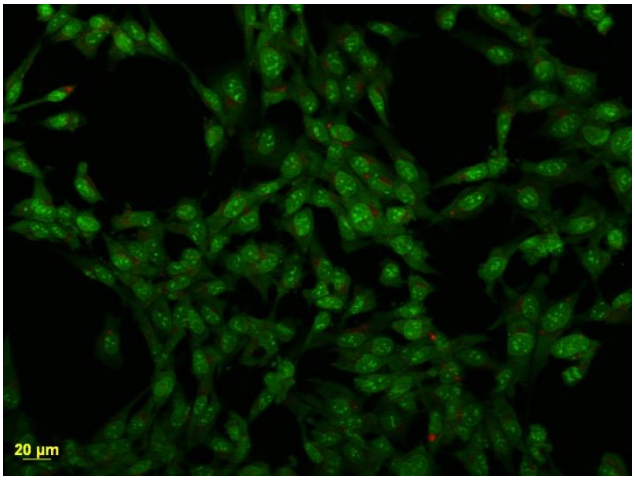


Figure 4.2. Epifluorescent microscopy images of 3T3 cell monolayers after 72 hours in culture. The cells in (a) were incubated in normal growth medium, while (b) and (c) were incubated in medium containing enalapril 1 mM and captopril 1 mM respectively. Cells were stained with 5-Carboxyfluorescein diacetate and ethidium bromide before images were captured with a Zeiss AxioImager Z1 wide field fluorescent microscope and digital imaging system.

a)



b)



c)

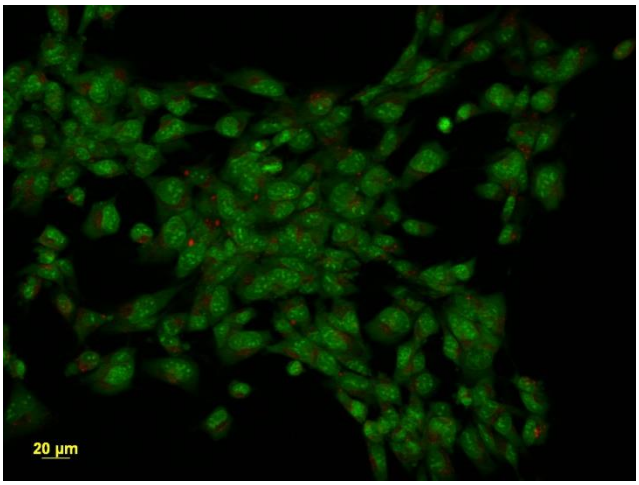


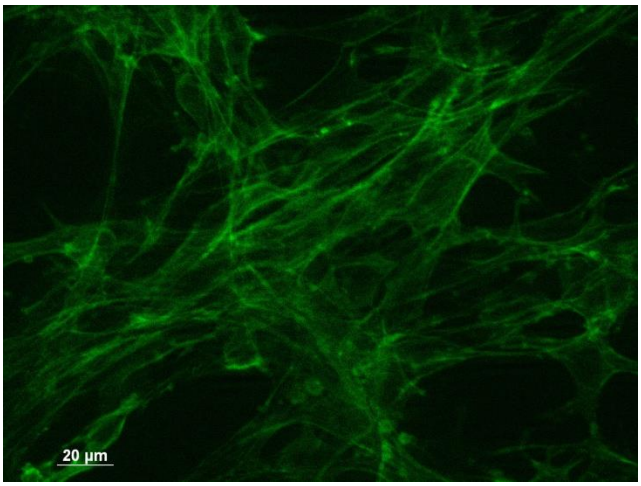
Figure 4.3. Epifluorescent microscopy images of 3T3 cell monolayers after 72 hours in culture. The cells in (a) were incubated in normal growth medium, while (b) and (c) were incubated in medium containing enalapril 1 mM and captopril 1 mM respectively. Cells were stained with acridine orange and propidium iodide before images were captured with a Zeiss AxioImager Z1 wide field fluorescent microscope and digital imaging system.

4.3.1.5 Staining of FPCLs with Phalloidin-FITC and PI

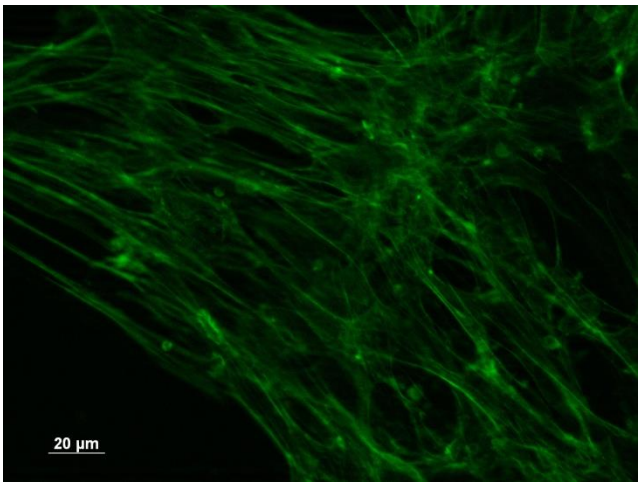
Epifluorescence microscopy images suggest that cells in collagen gels produce and distribute actin filaments as expected after 72 hours in culture both in the presence or absence of enalapril or captopril in the gels *(Fig. 4.4).* A green colour here indicates the presence and the position of actin filaments, and it can be noted for each of the different samples that the general structure of the filaments was similar, as was the manner in which they spread through the substrate and align themselves.

In addition to green stained actin filaments, the images in *Fig. 4.5* also have the cell nuclei stained in red. These images show that in the FPCLs containing enalapril or captopril cells retained a similar morphology to cells in untreated FPCLs. The images also suggest that after 72 hours in culture the cells in the ACE inhibitor-treated FPCLs were generally healthy and retained similarly high levels of viability to those in untreated FPCLs, with little observed difference between the overall levels of fluorescence in the control sample and the ACE inhibitor treated samples. In particular, *Fig. 4.5 (b)* clearly shows a cell undergoing mitosis within an enalapril treated FPCL, showing that cell division was still taking place. There was also little evidence of cell blebbing or apoptosis in any of the samples, and the nuclei were intact.

a)



b)



c)

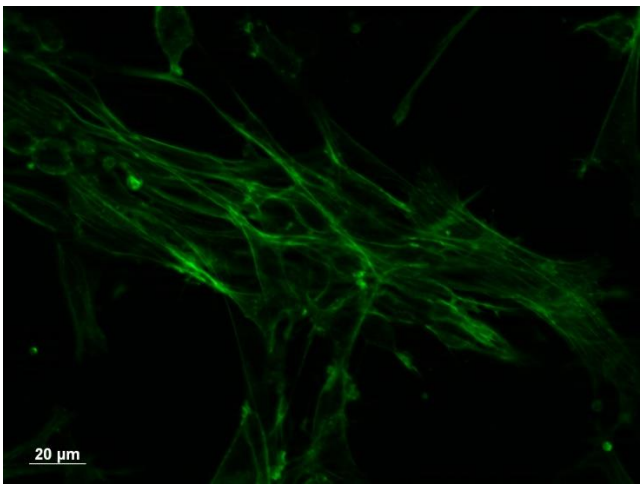


Figure 4.4. Epifluorescent microscopy images of 3T3 cells within collagen gels after 72 hours in culture. The cells in (a) were cultured in untreated collagen gels, while (b) and (c) were cultured in gels containing enalapril 1 mM and captopril 1 mM respectively. Actin filaments were stained with phalloidin-FITC before images were captured with a Zeiss AxioImager Z1 wide field fluorescent microscope and digital imaging system.

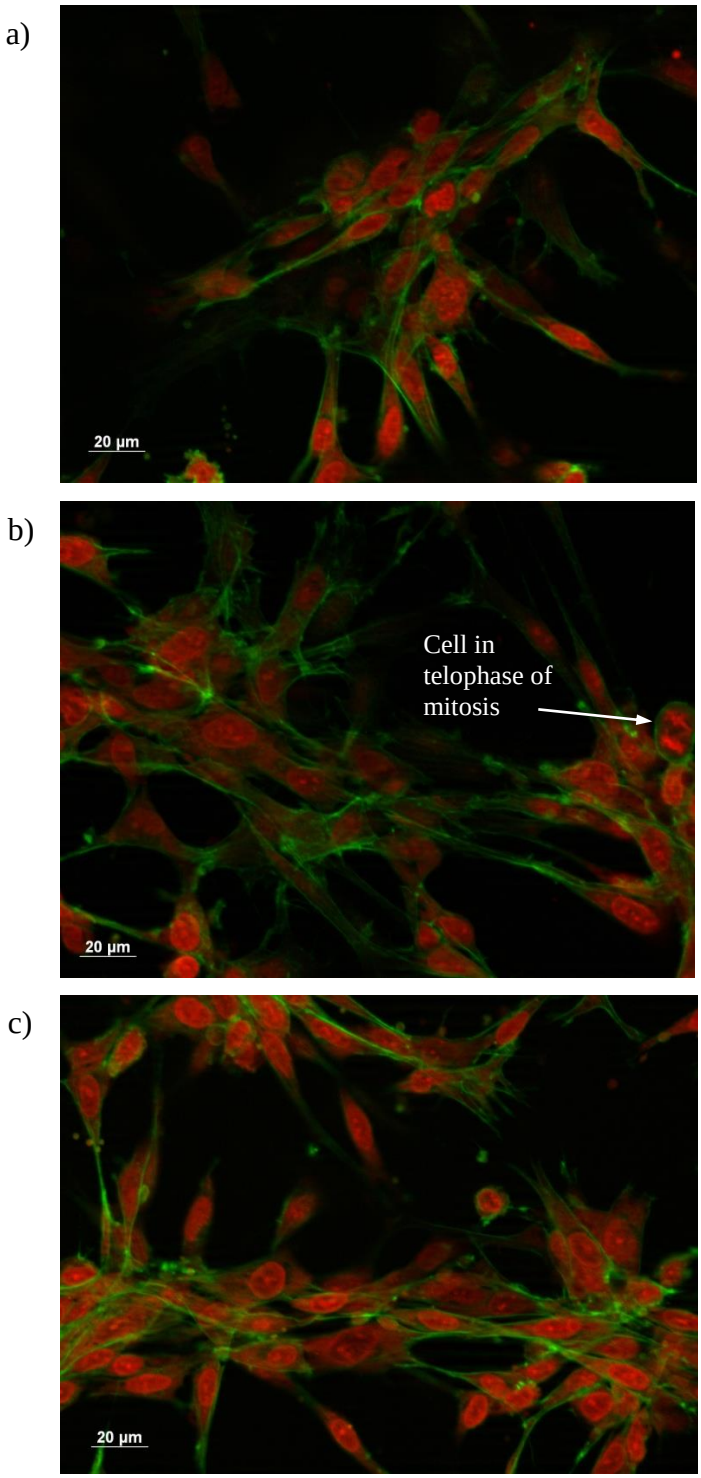


Figure 4.5. Epifluorescent microscopy images of 3T3 cells within collagen gels after 72 hours in culture. The cells in (a) were cultured in untreated collagen gels, while (b) and (c) were cultured in gels containing enalapril 1 mM and captopril 1 mM respectively. Actin filaments were stained with phalloidin-FITC and cell nuclei stained with propidium iodide before images were captured with a Zeiss AxioImager Z1 wide field fluorescent microscope and digital imaging system.

4.3.2 Contraction of FPCLs

The contraction study showed that FPCLs containing enalapril 1 mM or captopril 1 mM followed a similar pattern of contraction over a period of 10 days to untreated FPCLs *(Fig. 4.6)*. The untreated FPCLs contracted from 23.13 ± 0.33 cm^2 to a minimum of 5.68 ± 0.93 cm^2 on day 10, while results for enalapril and captopril treated FPCLs were similar with contractions of 22.78 ± 0.32 cm^2 to 6.50 ± 0.99 cm^2, and 23.56 ± 0.08 cm^2 to 5.40 ± 0.89 cm^2 respectively. As expected the PUV treated samples showed little variation in size throughout the 10 day period, starting at 22.66 ± 0.27 cm^2 and finishing at 22.60 ± 0.17 cm^2. It was also noted that cells did not appear to proliferate following treatment with PUV. Two-way ANOVA followed by Dunnett's test demonstrated no significant statistical differences between the contractile profiles of the negative control and the FPCLs treated with enalapril ($p = 0.148$) or captopril ($p = 0.994$), while the profile of FPCLs treated with PUV demonstrated large statistically significant differences compared to all other samples ($p < 0.001$).

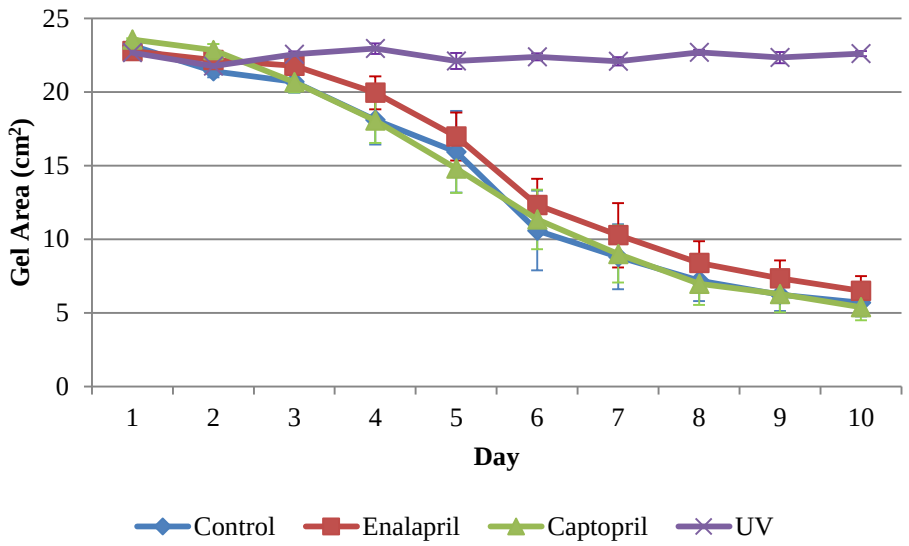


Figure 4.6. Ability of 3T3 cells to contract FPCLs, ascertained over 10 days. Negative controls were untreated FPCLs, while positive controls were treated with PUV light on day 1 to halt cell proliferation. As indicated, other FPCLs contained enalapril 1 mM or captopril 1 mM. Results are mean ± SEM, n = 4, and were compared by two-way ANOVA followed by Dunnett's test with respect to control samples.

4.4 Discussion

4.4.1 Cell Viability Studies

Past research into the pharmacokinetic and pharmacodynamic profiles of both enalapril and captopril have shown that, following therapeutic administration of the drugs for treatment of hypertension, blood serum levels of the drugs and their metabolites are unlikely to exceed 100 nM for enalapril (Biollaz *et al*, 1982; Johnston *et al*, 1984) or 1100 nM for captopril (Al-Furaih *et al*, 1991). However, the results presented in **chapter 3** suggest that concentrations of enalapril or captopril approaching 1 mM may be required to achieve marked inhibition of collagenolytic enzymes. Therefore it was appropriate to perform toxicity testing at these relatively high ACE inhibitor concentrations in order to ascertain whether the incorporation into cell-seeded constructs of enalapril or captopril at concentrations that could inhibit collagenase would also permit retention of cell viability and function.

4.4.1.1 Microplate Assays for Measurement of Cell Viability

Collagen digestion experiments had shown that for both enalapril and captopril marked levels of collagenase inhibition could be achieved at concentrations approaching 1 mM (see **section 3.3**). It was therefore decided to perform toxicity testing of cell monolayers in the presence of up to 1 mM of each drug in growth medium.

MTT and NR assays showed the cells to remain viable at concentrations necessary for collagenase inhibition. In some cases it was even evident that the presence of enalapril or captopril can have a positive effect on cell numbers and activities. Although the NR assay did not suggest any significant differences for concentrations of inhibitors below 1 mM, the MTT assay did show a significant *increase* in absorbance ($p < 0.001$) at 100 µM captopril. It is hypothesised that this increase in absorbance could be the result of cells effectively overcompensating in response to the presence of an unfamiliar foreign agent by increasing their metabolic activity. This theory of increased metabolic activity is in keeping with the more pronounced

positive effect on cell viability suggested by the MTT assay (when compared with the NR assay) as the reduction of MTT to formazan is an indicator of metabolic activity rather than simply a measure of viability.

The only significant negative effects on cell viability were observed at the highest ACE inhibitor concentration tested (1 mM), where both assays still reported greater than 80% viability for either drug, suggesting that enalapril and captopril both have little toxic effect to cells in culture, even at relatively high concentrations. Such low levels of apparent toxicity are reassuring considering these are drugs that have been widely prescribed for many years to treat patients suffering from hypertension.

4.4.1.2 Epifluorescence Microscopy Analysis of 3T3 Fibroblasts Cultured With High Concentrations of Enalapril and Captopril

Although CFDA and EtBr were used initially for cell staining, the use of these stains resulted in increased levels of cell blebbing, even in the control samples. At first it was thought that these high levels of blebbing were most likely attributable to the method used for staining cells with CFDA, which involved a step where cells were incubated at 4 ºC for 5 min. It was suspected that this cold incubation stage, although relatively short, was inducing apoptosis in the cells. However, it was later realised that other researchers were experiencing similarly unexpected blebbing effects when using CFDA, and it is now assumed that difficulties experienced were most likely due to problems with the specific batch of CFDA and not the technique itself.

AO and PI were then chosen for staining cell monolayers as these agents would also give an indication of cell viability while allowing cell shape and morphology to be viewed, but did not appear to have any toxic effect to the cells. The resulting images suggest that after 3 days in culture cells retain high levels of viability and are generally healthy, even in the presence of high concentrations (1 mM) of enalapril or captopril in growth medium. There were no noticeable differences in cell shape or morphology between the samples, and little evidence of cell blebbing or apoptosis, further implying that the ACE inhibitors have little toxic effect to cells in culture at

concentrations which can also inhibit the action of collagenase. Although there was a small amount of red fluorescence visible in all the samples (implying dead or dying cells), the level of red fluorescence in the ACE inhibitor treated samples did not appear any greater than in the control samples, implying that any cell death was likely not attributable to the presence of the ACE inhibitors. There was also little observed difference between the levels of green fluorescence in the control samples and the ACE inhibitor treated samples, further suggesting that the presence of enalapril or captopril was having little detrimental effect on the viability of the cell monolayers.

Since one of the main aims of this research was to incorporate enalapril and captopril into FPCLs, it was also important to check the viability of cells within collagen hydrogels that had been pre-treated with the ACE inhibitors. Cells in 3D culture are known to behave differently compared with cell monolayers (Petersen *et al*, 1992), and it has been suggested that the ability of cells to migrate within a matrix and remodel it depends greatly upon the physical and biochemical characteristics of the matrix (Even-Ram & Yamada, 2005). It was therefore decided that, in addition to staining cells in order to view their shape and morphology, it would also be advantageous to view the associated actin filaments as these structures are integral to the ability of fibroblasts to migrate and to remodel the substrate. Epifluorescence microscopy of FPCLs stained only with phalloidin-FITC suggest that the presence of enalapril or captopril at concentrations necessary for collagenase inhibition does not affect the ability of cells within the matrix to produce and distribute actin filaments as normal. Microscopy images indicated that the general structure of actin filaments in ACE inhibitor treated FPCLs did not vary noticeably from those in the control FPCLs, and that the manner in which they migrate through the substrate and align themselves was also unaffected by the presence of the ACE inhibitors, suggesting that enalapril and captopril should have little effect on the way in which cells contract and remodel the gel substrate (see **section 4.4.2**). FPCLs stained with both phalloidin-FITC and PI allowed for the cells within the matrix to be viewed in addition to the actin filaments, and images suggested that after 3 days culture in the presence of high concentrations (1 mM) of enalapril or captopril cells in collagen hydrogels remained healthy and viable and their general morphology was unaffected,

with little evidence of blebbing or apoptosis. Of particular note is the imaging of cells in the telophase of mitosis (latter stage of cell division) in an enalapril treated FPCL, demonstrating that cell division is still taking place and furthering the notion that cell viability is relatively unaffected by the presence of high concentrations of ACE inhibitors within collagen gel substrates.

All the results from epifluorescence microscopy studies combine to demonstrate that concentrations of enalapril or captopril that can inhibit collagenase have little or no detrimental effect to the viability of cells in 2D monolayers or on 3D collagen hydrogels. The microscopy images also suggest that cells remain proliferative and are able to retain normal functionality in the presence of these high ACE inhibitor concentrations.

4.4.2 Contraction of FPCLs

Contraction of the ECM by fibroblasts is an important physiological process, and FPCLs are commonly accepted as an appropriate model of wound contraction *in vitro* (Grinnell, 1994). Consequently, the contractile profiles of FPCLs were compared over a period of 10 days to ascertain whether the presence of enalapril or captopril would have any effect on the ability of the cells within the matrix to remain viable, function as normal, and contract the substrate.

It has been shown that angiotensin II and III in cardiac fibroblasts can actually stimulate contraction of collagen gels, although the mechanism by which this occurs is unknown (Lijnen *et al*, 2002), and accordingly it was hypothesised that the addition of ACE inhibitors to FPCLs may result in a reduction of matrix contraction as such drugs are primarily intended to block the conversion of angiotensin I to angiotensin II. However, such an effect was not evident, and it was observed that the FPCLs containing either ACE inhibitor followed a similar pattern of contraction to that of the untreated negative control over the 10 day study, contracting to approximately a quarter of their original size, suggesting that even after extended periods in culture cells remain viable and retain much of their functionality despite

the presence of high concentrations of enalapril or captopril. These findings supplement the results in **section 4.3.1.5** which show actin filaments and cell morphology in FPCLs to be unaffected by high concentrations of ACE inhibitors, in turn suggesting that the cells should retain their contractile properties.

While the contractile profiles of the untreated and ACE inhibitor treated FPCLs did not differ significantly, the PUV treated FPCLs did not show any evidence of contraction, demonstrating that contraction of the other samples could be attributed solely to the action of the fibroblasts within the matrix. These results also confirm that treatment with PUV is an effective way in which to halt cell proliferation and consequently to halt the contraction of FPCLs.

4.4.3 Conclusions from Cytotoxicity Studies

Toxicity testing of fibroblasts in the presence of enalapril and captopril has confirmed that neither ACE inhibitor is particularly toxic to 2D or 3D cultures, even at concentrations beyond those required for marked inhibition of collagenase. It has also been noted that in some cases the presence of ACE inhibitors could actually increase the metabolic turnover of cells in culture leading to increased proliferation. It is of particular note that cells in FPCLs containing high concentrations of enalapril or captopril are unaffected in terms of viability, morphology and actin distribution, and that the presence of the ACE inhibitors does not affect the ability of cells to remodel and reshape the substrate. It can therefore be assumed that any differences in mechanical characteristics of FPCLs with and without ACE inhibitors are more likely attributable to the action of the drugs on collagenolytic enzymes than to differences in the mechanical actions of cells within the matrix.

Chapter 5

Mechanical Characterisation of Collagen Hydrogels: Part I

5 Mechanical Characterisation of Collagen Hydrogels: Part I

5.1 Introduction

One of the main aims of this body of research was to determine means to manipulate and control the stiffness of cell-seeded collagen-based matrices. It was therefore necessary to find ways of testing mechanical characteristics of such matrices in order to differentiate between scaffolds of varying mechanical properties. This chapter is concerned with the design and validation of a method and an entirely novel device for the mechanical characterisation of sterile collagen hydrogels.

5.1.1 Device for Sterile Confined Compression of Hydrogels

The majority of techniques for characterising soft hydrated tissues are destructive or are at least not suitable for the repeated testing of sterile, cell-seeded samples (see **section 1.4.2.1**). It would be of great benefit if a method could be developed to allow for non-destructive, repeated testing of the same samples over a timeframe of days or even weeks in order to determine how their mechanical characteristics are affected relative to their time in culture. It would also be advantageous if the technique allowed for relatively fast, high throughput sample testing.

Following an assessment of the many various methods that are employed for the mechanical characterisation of biological tissues and the inherent difficulties associated with characterising highly hydrated tissues (see **section 1.4.2)**, a decision was made to focus on and adapt the technique of confined compression. By utilising confined compression, collagen hydrogels could be tested in the wells of standard tissue culture plates (24-well), meaning that they would not need to be moved prior to testing, facilitating the requirement to maintain sterility. Furthermore, collagen hydrogels may be considered biphasic in their construction, consisting of a loosely connected network of collagen fibrils (solid phase) filled with a large excess of interstitial fluid (fluid phase, typically > 99%), and confined compression allows for the analysis of this biphasic behaviour (Mow *et al*, 1980). While unconfined compression could also be used to analyse the biphasic behaviour of hydrogels, their

weak structure may mean that some kind of lateral support is required to prevent them from deforming under their own weight.

It was hypothesised that by constructing a tight-fighting lid for the culture plates made entirely of autoclavable materials, sterility could be maintained for testing. The lid would have individual porous indenters for each well of the plate, and linear guides for the indenters that should provide a sufficient seal to maintain sterility while also being minimally susceptible to friction.

5.1.2 Biphasic Theory

While the main requirement was simply to be able to differentiate between hydrogels of varying mechanical characteristics, it was also desirable to obtain further meaningful parameters that would provide greater insight into the material properties and allow for direct comparisons with other biological tissues. Biphasic theory (Mow *et al*, 1980) has often been used to characterise cartilaginous tissues (Ateshian *et al*, 1997; Iatridis *et al*, 1998; Périé *et al*, 2005), which are broadly similar in composition to collagen hydrogels in that they consist primarily of a collagen fibril network and interstitial water, although the collagen content in cartilaginous tissues is far greater. It was therefore hypothesised that a biphasic theory for intrinsically incompressible mixtures (Holmes, 1986; Mow *et al*, 1984; Mow *et al*, 1980) may also be suitable to analyse the material behaviour of collagen hydrogels. This theory assumes that the tissue is a mixture of an intrinsically incompressible and elastic solid phase and an intrinsically incompressible and inviscid fluid phase, where time-dependent effects are attributed to momentum exchange between the solid and fluid phases due to frictional drag. This theory is essentially equivalent to Biot's poroelasticity theory as developed in the field of soil mechanics (Biot, 1956; Biot, 1973).

A biphasic theory for mechanics of tissue equivalents proposed by Barocas & Tranquillo (1997) has previously been applied to collagen hydrogels in confined compression (Knapp *et al*, 1997), although this particular theory also includes terms for shear modulus, viscosity and Poisson's ratio, and therefore can only be applied in

conjunction with data from rheological and unconfined compression testing. In contrast, the linear biphasic theory of Mow *et al* (1980), allows for estimation of the aggregate modulus (Young's modulus in confined compression) and hydraulic permeability of a material in confined compression – based solely on the stretch ratio (λ) and corresponding stress response – by non-linear regression of the numerical solution for Eq. (1).

$$\frac{\partial^2 u}{\partial z^2} = \frac{1}{H_A k} \cdot \frac{\partial u}{\partial t} \tag{1}$$

where u is the displacement of the solid phase in the direction z and at time t, H_A is the aggregate modulus, and k is the hydraulic permeability. While later models have also introduced terms for non-linear elasticity (Ateshian *et al*, 1997; Périé *et al*, 2006), it has been suggested that the equilibrium elastic response of intervertebral disc tissue is better described by the linear model at low strain amplitudes (Périé *et al*, 2005). Indeed, Barocas *et al* (1995) demonstrated that the collagen stress response is linear up to ~10% strain. Consequently, since low strain amplitudes were adopted for the confined compression experiments with collagen hydrogels, the linear biphasic model of Mow *et al* (1980) was used to calculate H_A. In this infinitesimal (small strain) theory the slope H_A of the stress-stretch (or stress-strain) curve remains constant. However, it has also been suggested that permeability decreases with compressive strain even at such low strain amplitudes (Périé *et al*, 2005), i.e. under compression the network of collagen fibres becomes more dense making it more difficult for fluid to permeate through it. The hydraulic permeability, k, was related to the stretch ratio, λ, using Lai & Mow (1980):

$$k(\lambda) = k_0 e^{M(\lambda - 1)} \tag{2}$$

where k_0 is the zero-strain hydraulic permeability, and M is a non-dimensional non-linear permeability coefficient describing the loss of permeability with compression. The stretch ratio, λ, is the ratio of the sample height to its original height, and is defined as:

$$\lambda = 1 + \varepsilon \tag{3}$$

where ε is the strain ($\partial u/\partial z$). By using the above model it may be possible to directly compare the biphasic parameters obtained for collagen hydrogels with those calculated for other collagenous biological tissues where similar models were employed (Ateshian *et al*, 1997; Périé *et al*, 2005).

5.2 Materials and Methods

A device was designed and manufactured for the purposes of characterising collagen-based hydrogels. The device consists of an autoclavable tight-fitting lid with integrated porous compression platens that fits on a standard 24-well tissue culture plate *(Fig. 5.1(a))*. Initial versions used lids constructed of polytetrafluoroethylene (PTFE), but it was quickly realised that this material was unsuitable (see **section 5.4.1**) and subsequent versions were constructed in aluminium. Each of the compression platens consists of a narrow stainless steel shaft supporting a circular porous indenter (316L sintered steel mesh) of diameter ~15.9 mm *(Fig 5.1(b))*. A removable disc of ferrous metal was screwed to the top of the shaft allowing for attachment to a 1 kN load cell (Zwick/Roell, UK) via a magnet, though earlier versions of the device incorporated springs to support the platens. While the early prototypes simply used holes drilled through the aluminium for shaft guidance, later versions incorporated linear rolling-element bearings, eventually leading to the use of Drylin® linear bearings (Igus®, UK), which have no moving parts and are suitable for autoclave sterilisation. The device was intended to enable the indirect determination of the mechanical properties of tissue samples via confined compression and biphasic theory (Lai & Mow, 1980; Mow *et al*, 1980), while maintaining sterility.

All collagen gel samples for compression testing were 16 mm diameter and approx. 5 mm thick (1 ml total volume). Preliminary studies were concerned with confirming the suitability of the device and defining a protocol for testing hydrogels, and a variety of test conditions were assessed. 0.3% w/v collagen gels were compressed by between 5% and 8%, at 0.01 to 0.5 mm/s, and held for 60 to 300 s, with the test being repeated multiple times where appropriate.

To validate the theory and device, collagen gel samples were made in each of three collagen concentrations (0.2%, 0.3% and 0.4%). The surfaces of the gels were found by lowering the platen until a pre-load of 0.2 N was achieved, and then resting the system until the stress response equilibrated. Samples were then compressed by 250 µm (~5% strain) at 50 µm/s and held for 120 seconds. Biphasic theory (Ateshian *et al*, 1997; Mow *et al*, 1980) was fitted to the hold phase using a Nelder-Mead scheme (bespoke Matlab code provided by Dr. Phil Riches, University of Strathclyde (Riches, 2011)) and the tissue parameters H_A, k_0 and M, in addition to the peak and equilibrium stresses, were inversely engineered and subsequently compared using ANOVA.

a)

b)

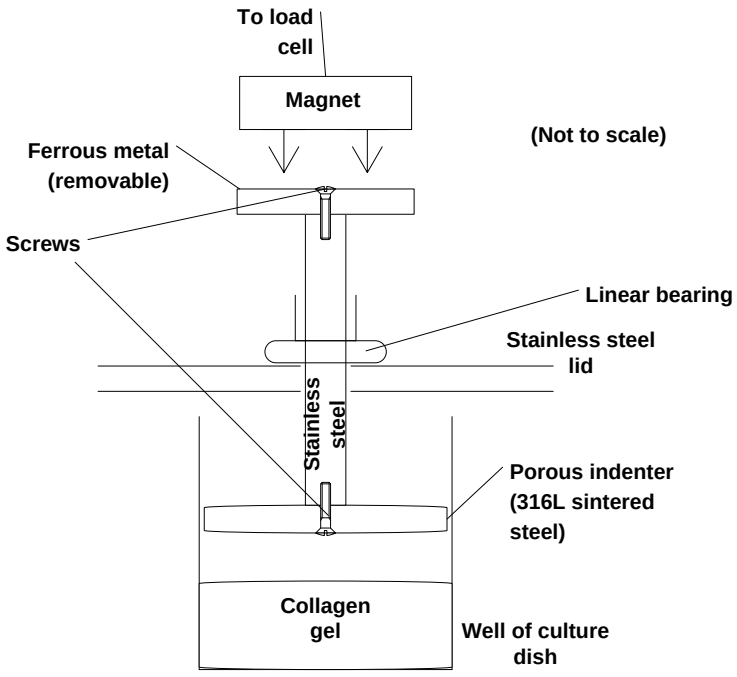


Figure 5.1. a) Photograph of prototype device for mechanical characterisation of hydrogels during testing and b) diagrammatic representation of a single compression system within the device. Attachment to the load cells is made via a magnet, though earlier versions used springs to support the piston shaft.

5.3 Results

Initial prototypes of the device were constructed using PTFE, but the material was overly pliable and susceptible to relatively large amounts of stress relaxation during testing (results not shown), and consequently further versions were constructed in aluminium.

Where springs were used to support the platens the stresses recorded during compression were relatively high, although it was possible to distinguish between the stress responses where collagen gels were or were not present in the tissue wells during testing *(Fig. 5.2(a))*. However, rather than observing a typical stress relaxation response following compressions the recorded output appeared to fluctuate erratically before levelling off. The stiffness of the springs also appeared to lessen with repeated testing, with lower stresses being recorded for subsequent compressions *(Fig. 5.2(a))*.

Modifying the device to use magnets to affix the platens to the force transducer, negating the requirement for springs, did result in a more typical stress relaxation response following compression *(Fig.5.2(b))*. Furthermore, the magnetic support itself did not contribute to the stiffness in compression and there was no perceived reduction in recorded stresses upon repeated testing, although there were still issues with friction in the system causing unwanted noise and enhanced stress readings even when tissue wells contained only growth medium *(Fig. 5.2(b))*.

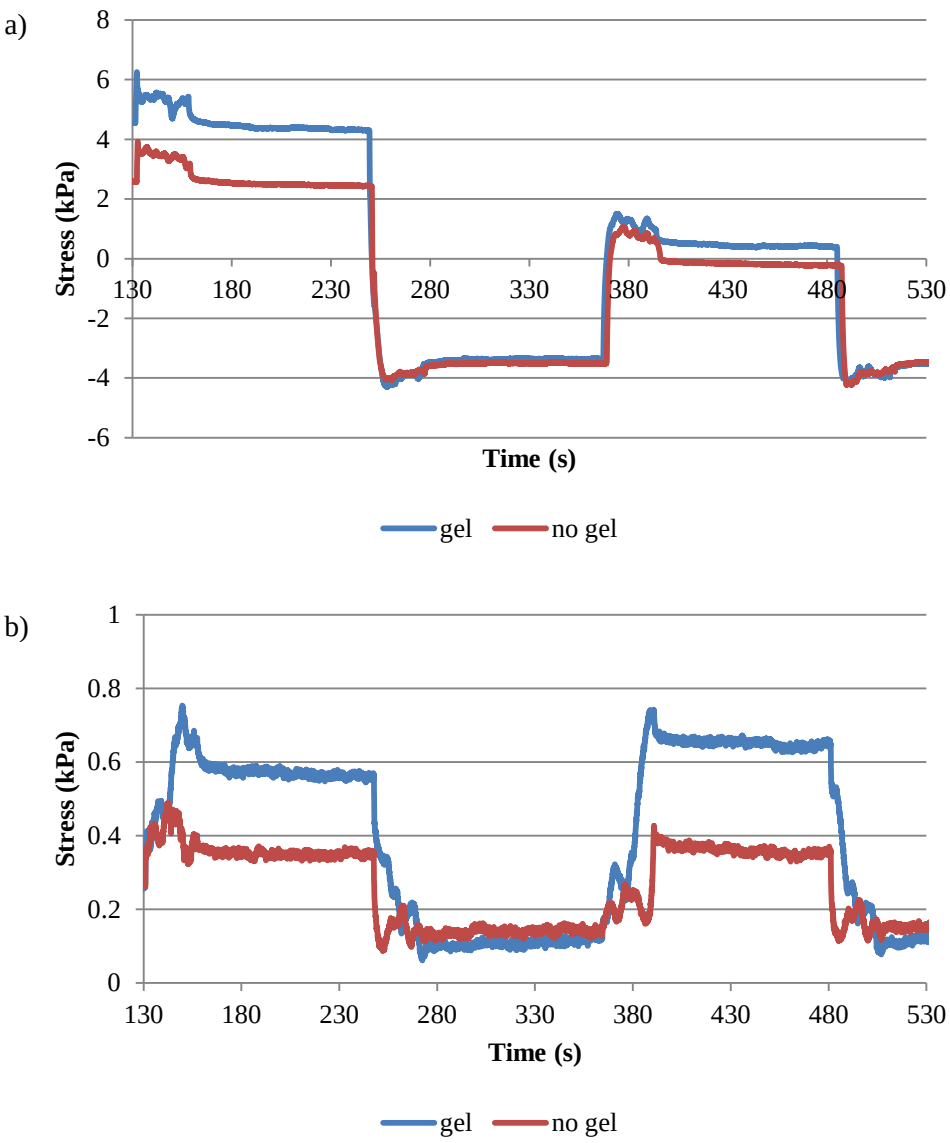


Figure 5.2. Comparison of typical compressive stress responses from confined compression experiments with and without collagen gels in growth medium. For both figures the platens were moved to 5 mm from the bottom of the tissue wells, held for 120 s, then lowered a further 0.25 mm at a rate of 10 µm/s and held for a further 120 s before the process was repeated. (a) Platens were supported with springs, (b) platens were attached to the load cell via a magnet.

Modifying the device to include linear rolling-element bearings to guide the platen shafts, in addition to increasing the rate of compression, resulted in much less apparent noise, and biphasic stress responses that were completely typical of poroelastic materials in confined compression *(Fig. 5.3)*. It is also clear from *Fig.5.3* that increasing the rate of compression results in higher peak stress values and a more abrupt stress relaxation phase.

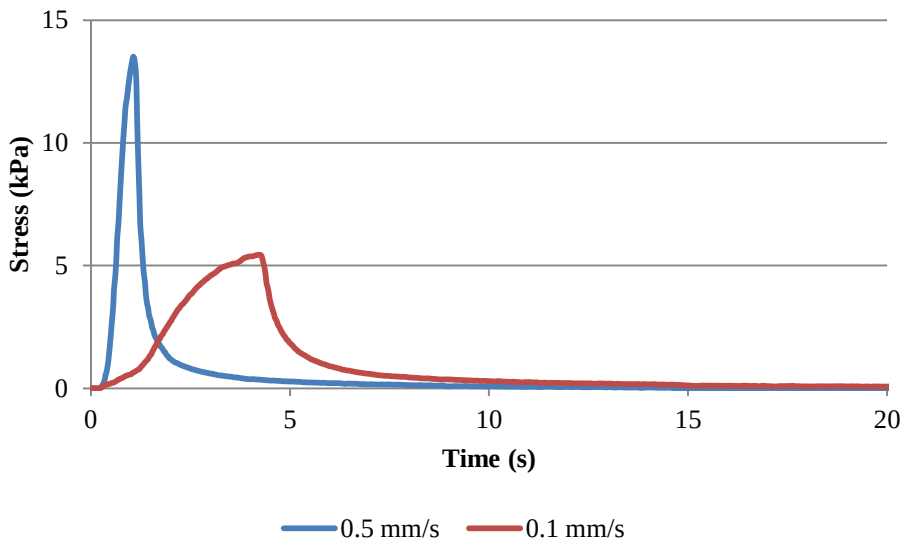


Figure 5.3. Compressive stress response from confined compression of collagen gels. Gels were compressed by 0.4 mm (~8%) at 0.1 and 0.5 mm/s and held.

At this stage a small number of tests were carried out to ascertain whether there were any major differences in the stress responses between gels tested submerged in growth medium or gels tested 'dry' i.e. with excess fluid removed prior to testing. Initial stress responses appeared similar for both the dry and submerged samples, although it was noted that when the platen was returned to its starting position and held for 120 s before repeating the test procedure the resulting peak stresses recorded were considerably lower than those observed from the initial compression *(Fig. 5.4(a))*. It is also clear from *Fig. 5.4(a)* that when the platen was returned to its starting position a considerable negative stress was induced, and this was far greater for the dry samples. In addition, it was noted that there were once again unwanted

frictional artefacts present during the actual compressive phase, especially at the very start of compression where there appears to be a considerable jump in the recorded stress (*Fig. 5.4(b)).*

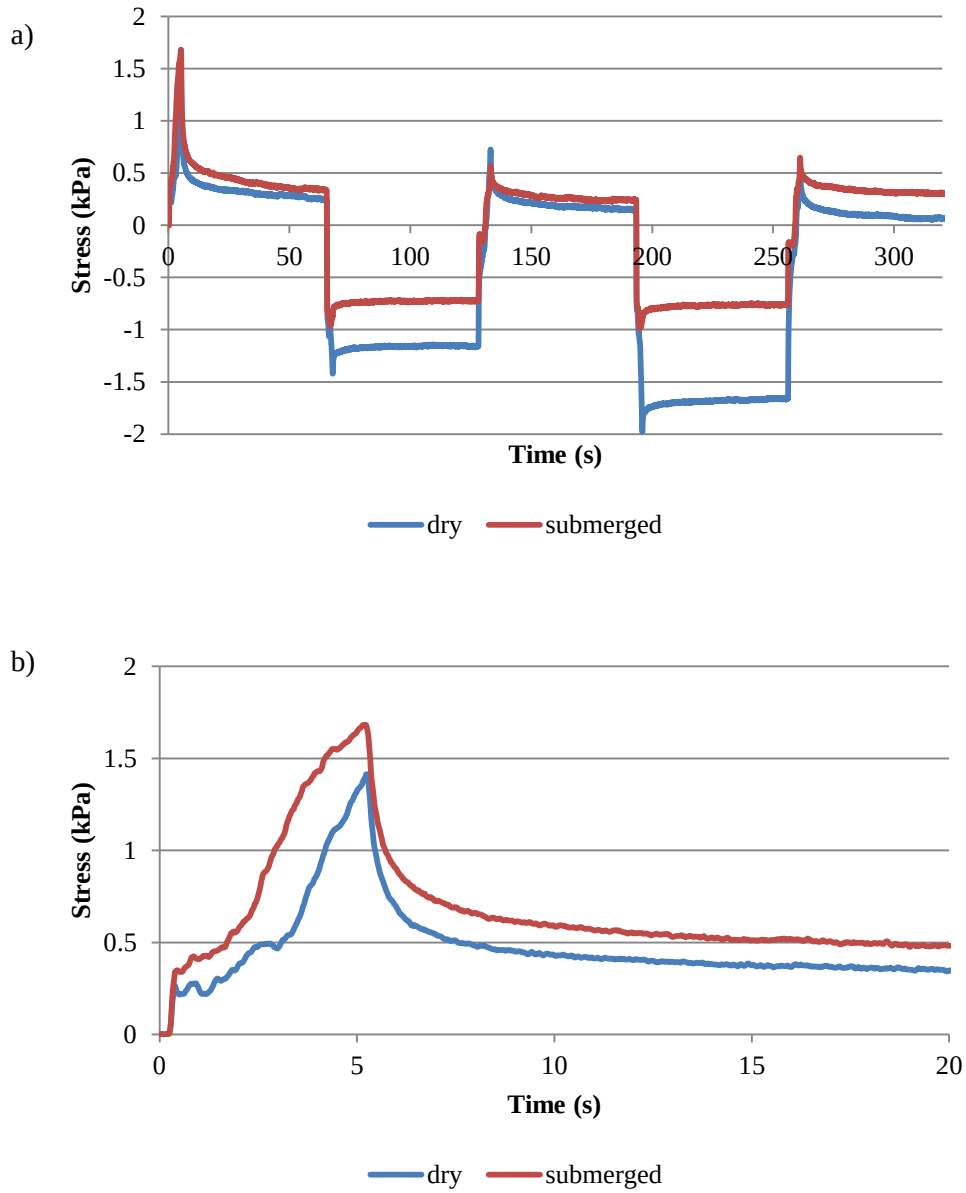


Figure 5.4. Comparison of typical compressive stress responses from confined compression of collagen gels either submerged in growth medium ('submerged') or with the excess fluid removed ('dry'). Platens were lowered until a pre-load of 1 N (4.97 kPa) was achieved and then held until the stress response equilibrated. Samples were then compressed by 250 µm at 50 µm/s and held for 120 s. The platen was then retracted 250 µm and held for a further 120 s before the compression/decompression process was repeated. Results are mean values, n = 2. Both figures show the same results (a) 0 to 320 s (b) 0 to 20 s.

In order to try and remedy the perceived reduction in sensitivity and increase in frictional artefacts observed following repeated use of the device, new linear bearings with a core of Drylin® were sourced as these had no moving parts. Early testing with the new linear bearings did indeed result in more smooth, less noisy stress responses for collagen gels in confined compression *(Fig. 5.5)*. The same collagen gels were tested multiple times in order to assess the effect of repeated compression tests, with the gels being allowed sufficient time to fully recover after each individual compression. *Fig. 5.5* demonstrates that with each subsequent compressions the peak stress increases ($p = 0.02$). There were no statistically significant differences in the equilibrium stress following the 120 s hold time after compression (not shown on *Fig. 5.5*). For all further experiments only a single compression test was performed on each hydrogel.

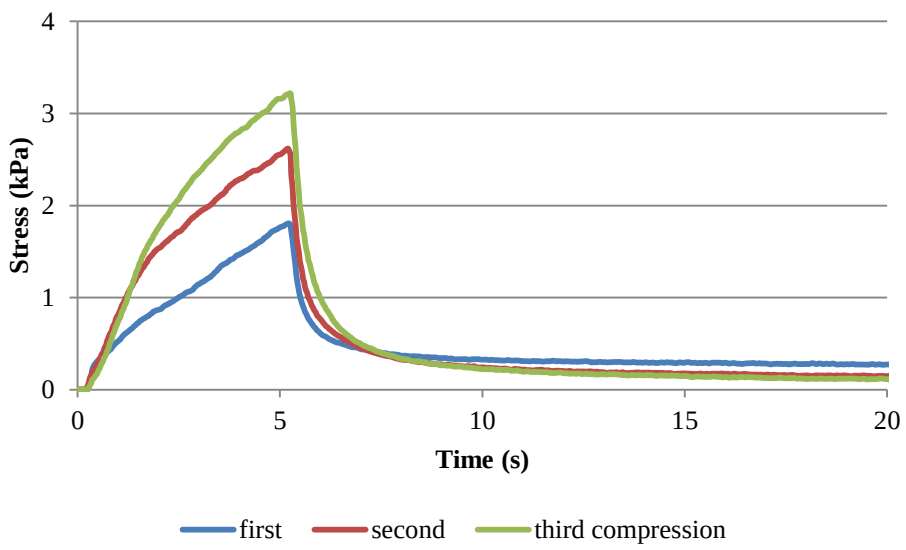


Figure 5.5. Average compressive stresses from repeated compression testing of collagen gels, plotted against time. Platens were lowered until a pre-load of 0.2 N (0.99 kPa) was achieved and then held until the stress response equilibrated. Samples were then compressed by 250 µm at 50 µm/s and held for 120 s (0 to 20 s shown in figure). Results are mean values, n = 3.

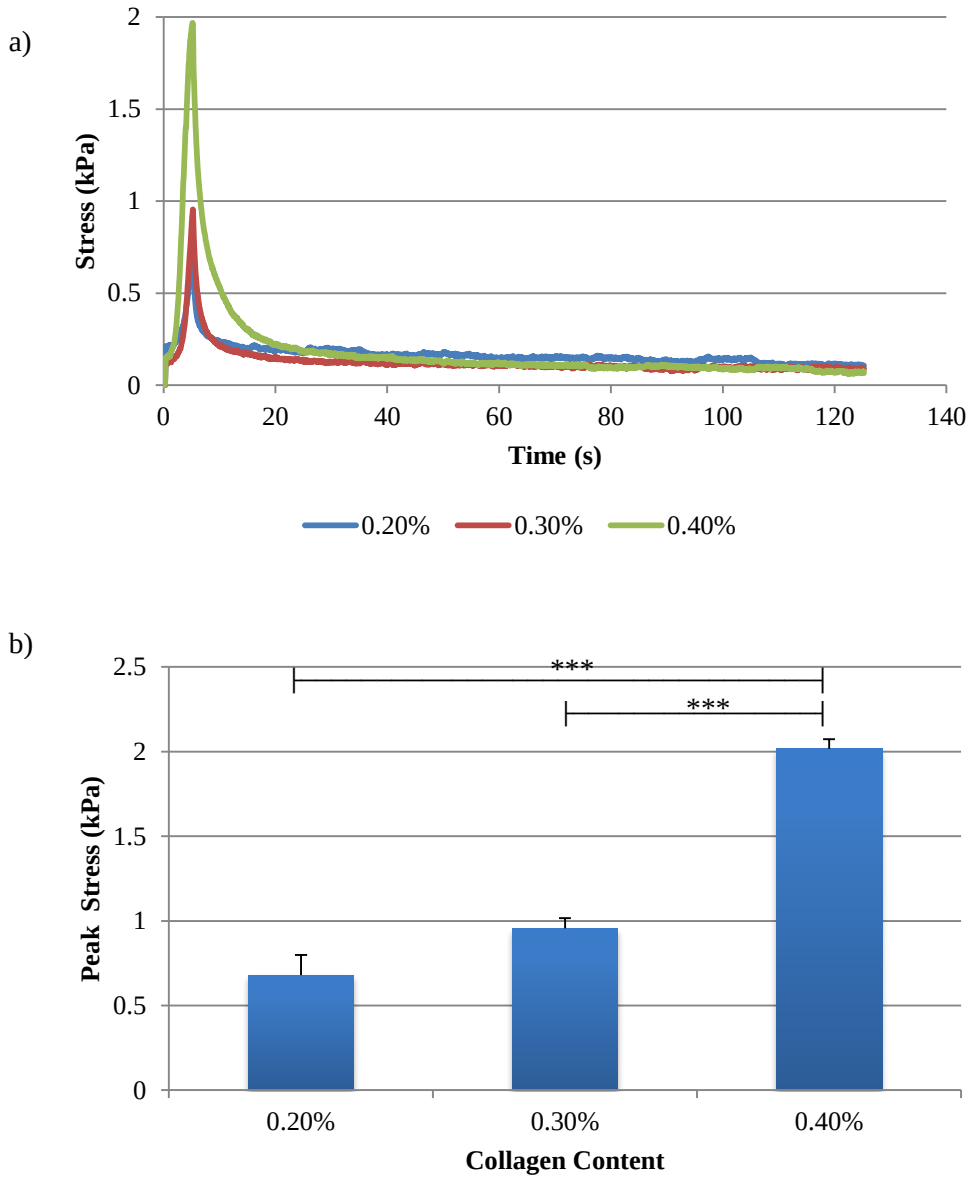


Figure 5.6. a) Average compressive stresses for hydrogels of three different collagen concentrations in confined compression, plotted against time. Platens were lowered until a pre-load of 0.2 N (0.99 kPa) was achieved and then held until the stress response equilibrated. Samples were then compressed by 250 µm at 50 µm/s and held for 120 s. b) Relationship between peak compressive stress and collagen content in hydrogels. Results are mean + SEM, n = 3, and were compared by ANOVA followed by Tukey's test. Significant variations: *** $p < 0.001$, 95% confidence.

Increasing the collagen content of the hydrogels resulted in significant increases in peak stress ($p < 0.001$) following confined compression *(Fig.5.6(b))*, though there was no variation in equilibrium stress. However, there was again evidence of new

frictional artefacts, specifically at the start of the ramp phase where there is again an obvious instantaneous increase in the recorded stress of around 0.2 kPa for all groups *(Fig. 5.6(a))*. Consequently, biphasic theory was only fitted to the hold phase, as is common in cartilage testing (Ateshian *et al*, 1997), and although none of the fitted parameters varied with collagen content, zero-strain hydraulic permeability showed a trend ($p = 0.069$) towards varying with collagen content *(Fig. 5.7)*. A power analysis suggested that significance would have been achieved if $n = 5$ for each group, and accordingly further tests were performed with a greater number of samples per group.

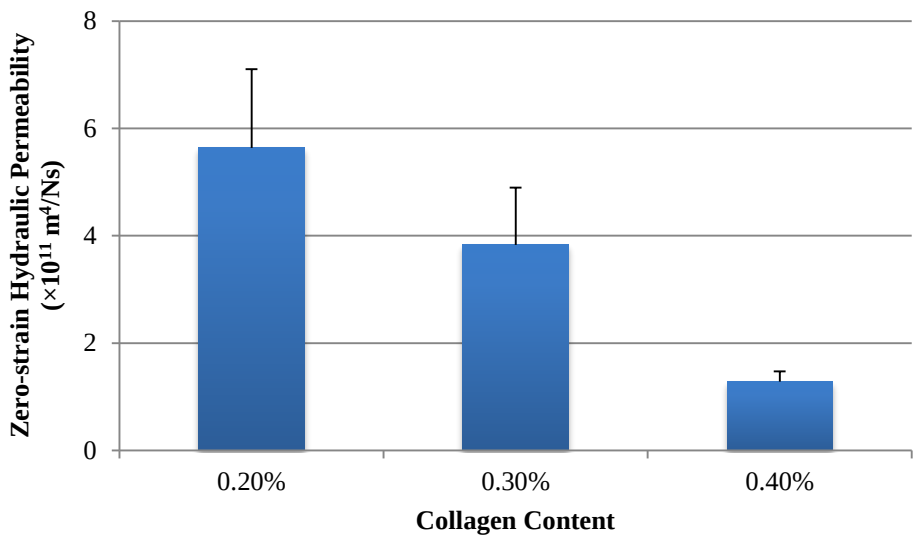


Figure 5.7. Relationship between the zero-strain hydraulic permeability and collagen content in hydrogels. Results are mean + SEM, n = 3, and were compared by ANOVA ($p = 0.069$).

Repeating the test procedure with $n = 8$ samples per group led to broadly similar results, and peak stress was the only variable that showed significant variation ($p < 0.001$), increasing relative to collagen content *(Fig. 5.8(b))*. The problem of an instantaneous increase in the stress response at the beginning of the ramp phase appeared worse than in previous tests, with an immediate jump to around 0.5 kPa for all groups *(Fig. 5.8(a))*. However, the stress relaxation following the ramp phase was again indicative of poroelastic (biphasic) behaviour. Consequently, the biphasic

equations (Lai & Mow, 1980; Mow *et al*, 1980), including strain dependent permeability, were able to model the behaviour efficiently.

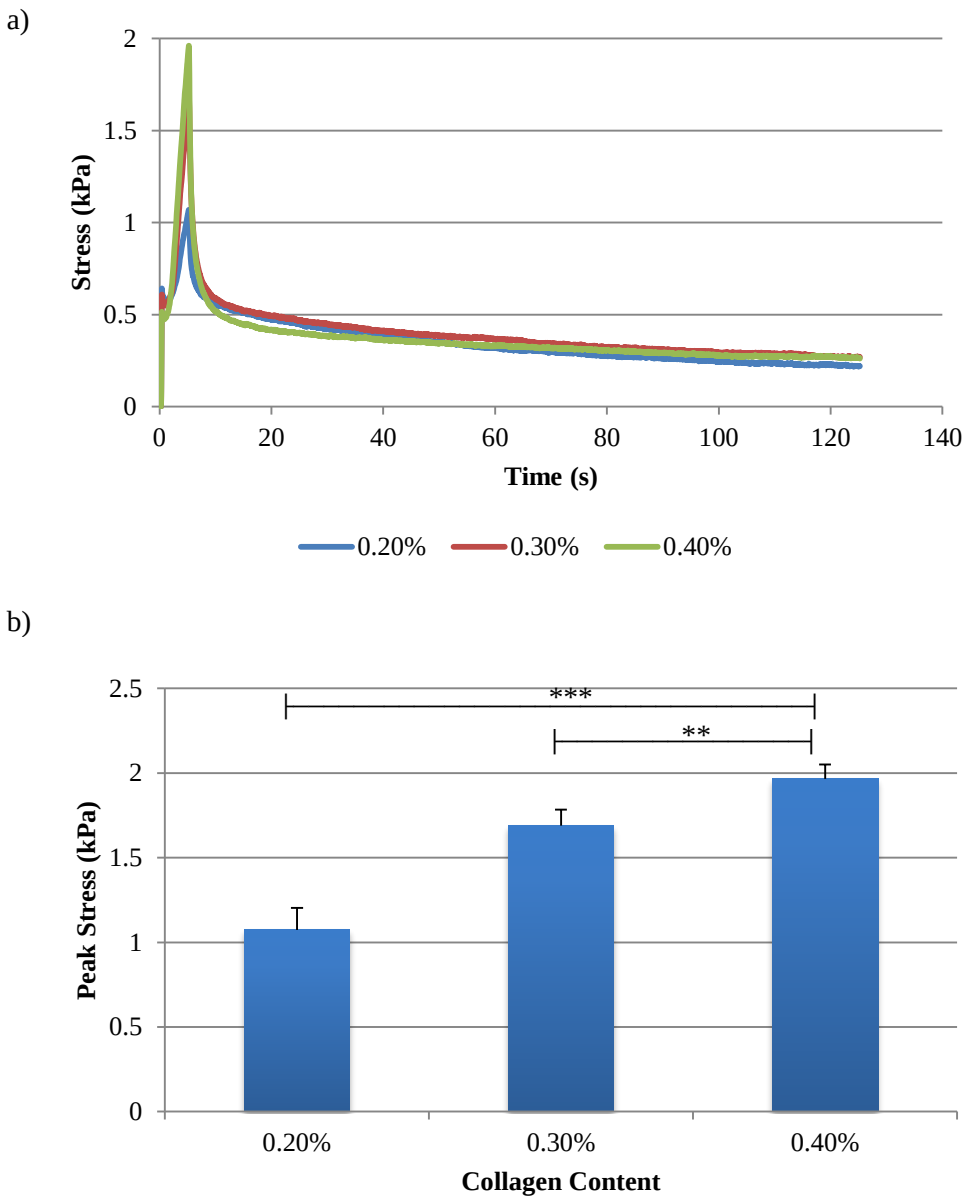


Figure 5.8. a) Average compressive stresses for hydrogels of three different collagen concentrations in confined compression, plotted against time. Platens were lowered until a pre-load of 0.2 N (0.99 kPa) was achieved and then held until the stress response equilibrated. Samples were then compressed by 250 µm at 50 µm/s and held for 120 s. b) Relationship between peak stress and collagen concentration in hydrogels. Results are mean + SEM, n = 8, and were compared by ANOVA followed by Tukey's test. Significant variations: ** $p < 0.01$, *** $p < 0.001$, 95% confidence.

Plotting the zero-strain hydraulic permeability against stiffness *(Fig. 5.9(a))* shows that as stiffness increases, permeability decreases (logarithmically), though this was not related to collagen concentration. Plotting the zero-strain hydraulic permeability against the nonlinear permeability coefficient *M (Fig. 5.9(b))* shows that as permeability decreases, the nonlinear behaviour of permeability increases.

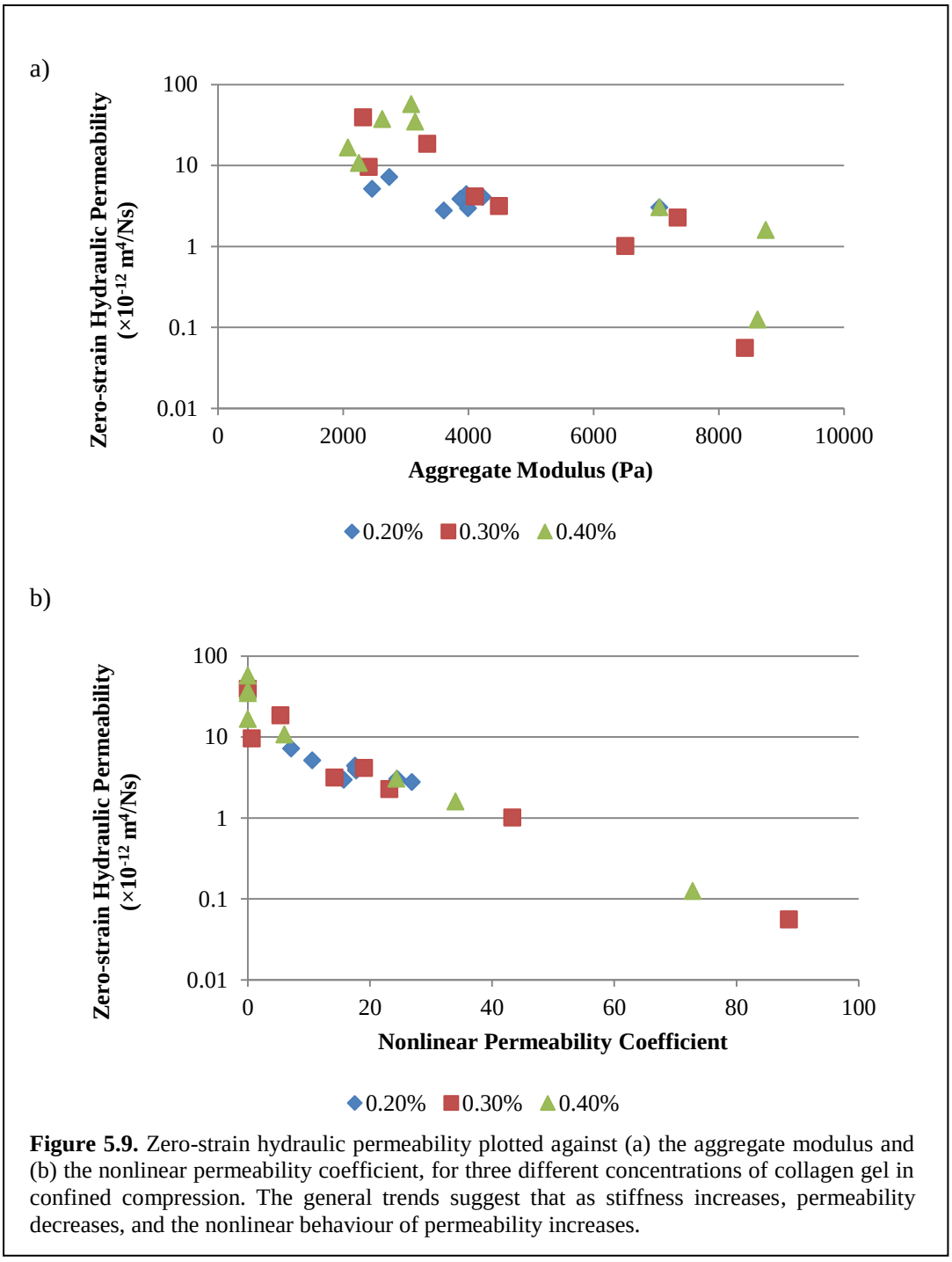


Figure 5.9. Zero-strain hydraulic permeability plotted against (a) the aggregate modulus and (b) the nonlinear permeability coefficient, for three different concentrations of collagen gel in confined compression. The general trends suggest that as stiffness increases, permeability decreases, and the nonlinear behaviour of permeability increases.

5.4 Discussion

Since one of the main aims of this research was to determine means to control the stiffness of collagen-based matrices, it was important to find ways to differentiate between matrices of varying mechanical properties. Although several methods have been proposed for the mechanical characterisation of collagen hydrogels, the majority of these are destructive or at least unsuitable for repeated tests of sterile samples, and only allow for extrapolation of a limited set of mechanical parameters (see **section 1.4.2.2**). Accordingly, attempts were made to design and implement a novel device and methodology that could be used to perform mechanical tests on the same samples at various time points, and to determine meaningful parameters to differentiate between samples of varying mechanical properties. The device was intended to consist of a tight-fitting lid made of autoclavable materials that would fit over standard 24-well tissue culture plates, with individual porous indenters corresponding to each well, that would allow for direct and indirect analysis of mechanical parameters of collagen hydrogels via confined compression in conjunction with biphasic theories for tissue-equivalent mechanics (Ateshian *et al*, 1997; Mow *et al*, 1980).

5.4.1 Design of Novel Device for Mechanical Characterisation of Collagen Hydrogels

PTFE was initially chosen for construction of the main components of the device due to its chemical stability, physical robustness, biological inertness and machinability (Chen *et al*, 2003). However, early tests using PTFE showed it to be overly pliable, with relatively large amounts of stress relaxation during compression testing being attributable to the material itself, and therefore it was concluded to be unsuitable for further use. Subsequent implementations were constructed of aluminium, which is much stiffer than PTFE, yet also highly machinable and suitable for sterilisation by autoclave.

The early versions of the device used springs to support the platens, and since the mechanical characteristics of springs are easily modelled it was assumed that the stress response of hydrogels in confined compression could be calculated by recording the overall stress response from compression of the platens and hydrogels together, and subtracting the stress response from the springs alone (tested separately under similar conditions). While there was a discernable difference in the stress responses where hydrogels were or were not present in the tissue wells, the expected biphasic stress relaxation pattern was not observed and suggested that the springs were likely to be causing a damping effect in the system, in addition to contributing to friction. The amplitude of the stress response from the springs also seemed to lessen considerably with subsequent compressions, suggesting that extensive preconditioning would likely be required if they were to be utilised.

By using magnets to attach the platens to the force transducer the need for springs (and therefore for preconditioning of the system) was eradicated, and a more typical stress response was observed following hydrogel compressions. However, there were still issues with friction and enhanced stress readings, and it was assumed that the rudimentary shaft guidance was responsible as this merely consisted of holes drilled directly into the aluminium lid. Accordingly, linear rolling-element bearings were added for shaft guidance, which when combined with an increased strain rate resulted in typically biphasic stress responses and minimal noise. It was noted that larger strain rates resulted in higher peak stresses and more abrupt relaxation phases, and although the increase in peak stress was expected (demonstrating strain rate dependence), the abruptness of the stress relaxation could be indicative of irreversible damage being done to the matrix. Therefore a strain rate was adopted for all further tests (50 µm/s) that was large enough to illicit a clear biphasic response, while not being so large as to cause discernible damage to the matrix or be susceptible to inertial effects.

Compression testing of hydrogels either fully submerged in growth medium or with all excess fluid removed immediately prior to testing demonstrated that the initial response was similar for both cases, suggesting that testing the hydrogels in fluid was not necessary. However, the hydrogels tested without excess fluid did induce a

considerable negative (i.e. tensile) stress when returned to their starting position, which was most likely caused by the gel sticking to the platen, adding to the overall weight. To reduce the potential problem of gels sticking, which could lead to increases in friction, all further testing with the device were performed with the hydrogels submerged in growth medium.

Returning the platen to the level which had originally been determined as the top surface of the hydrogel and holding it there for 120s before repeating the compression test resulted in markedly lower peak stresses. This was assumed to be a consequence of the gels requiring more time to recover and was considered inconclusive. In addition, a small jump in the recorded stress at the start of the ramp phase of compression was noted for all samples tested, suggesting friction in the system that had not been present previously, which could have been caused by deterioration of the linear bearings with repeated use. Although it was thought that lubricating the bearings may have alleviated the problem of friction, such an approach was not suitable as the lubricant would be likely to contaminate samples. Therefore new linear bearings were sourced with a core of Drylin®, as these have no moving parts, yet still allow near-frictionless shaft guidance. Indeed, early compression tests of hydrogels using these new linear bearings resulted in smoother stress responses than had been observed previously.

Earlier testing of the same gels multiple times had provided inconclusive results, and it was thought that the nature of the tests was the main issue, so more stringent test procedures were adopted. Allowing hydrogels more time to recover between individual compression tests demonstrated that with each subsequent compression the peak stress increased, implying that the test procedure itself was in fact having a measurable effect on the mechanical properties of the hydrogels. It was thought that the hydrogels may be experiencing an amount of permanent deformation, resulting in more densely packed collagen fibres and a less permeable matrix. It was therefore decided that when testing hydrogels only a single compression should be used to ascertain mechanical parameters, which would obviously have further implications if repeated tests on the same samples were to be performed over an extended period of time. However, with appropriate controls, such an approach would still be feasible.

Having completed manufacture of what was considered an acceptable prototype for the device, and designing a suitable protocol for compression testing of collagen hydrogels using it, efforts were then made to validate the efficacy of the device and technique in extrapolating mechanical characteristics of the hydrogels through the application of biphasic theory (Ateshian *et al*, 1997; Mow *et al*, 1980).

5.4.2 Validation of Device and Theory for Mechanical Characterisation of Collagen Hydrogels

In order to validate the device and the technique tests were performed on collagen hydrogels of three different collagen concentrations. Tensile testing of collagen hydrogels has previously shown that the linear modulus and failure strength of the matrices increases with collagen concentration (Roeder *et al*, 2002), and it was hypothesised that extracted parameters from confined compression of hydrogels would also vary with collagen content. In particular, it was hypothesised that as collagen content increases, stiffness should increase and permeability decrease.

During testing it was noted that, despite the incorporation of Drylin® linear bearings, there was a small jump in the recorded stress at the start of the ramp phase of compression, again suggesting that the system deteriorates and induces unwanted friction with increased usage. However, the stress relaxation response was again typical of poroelastic materials, and consequently biphasic theory was applied to the hold phase, allowing for estimation of the biphasic parameters H_A, k_0 and M, in addition to obtained values for the peak and equilibrium stresses. Initial tests with a small number of samples ($n = 3$ for each concentration) demonstrated that peak stress increased with collagen content ($p < 0.001$), suggesting that the method could be used to characterise mechanical differences in the hydrogels. However, there was no variation in the fitted parameters with respect to collagen content, although a trend was evident ($p = 0.069$) showing that k_0 decreased with collagen concentration. This statistical result is unsurprising given the low sample size, and following a power analysis it was concluded that repeating the tests with a greater number of samples should achieve statistically significant results for k_0 with respect to collagen content.

Unfortunately this was not the case, and even with n = 8 samples in each of the three collagen concentrations the results were broadly similar to those from the previous tests. This lack of significance may in part be accounted for by the perceived friction in the system.

Plotting k_0 against H_A showed that as stiffness increases, permeability decreases (logarithmically) as expected, although this was not related to collagen concentration. Furthermore, plotting k_0 against M showed that as the zero-strain permeability decreases, the nonlinear behaviour of permeability increases. The clear interplay among the parameters and the lack of association with collagen concentration highlights difficulties for the fitting of algorithms to achieve true specimen characterisation. The equation was fitted in multi-parameter space, and whilst the numerical method may find a numerically optimal set of parameters, these are not necessarily the true parameters of the sample (Riches, 2011). Therefore, the interdependence of M and k_0 may be indicative of a lack of uniqueness of the fitted parameters.

At this stage it was considered that the lack of significant variation of the biphasic parameters with respect to collagen content could be due to a number of factors. It was thought that the relatively small changes in collagen concentration (0.1%) with respect to fluid content may not have been sufficient to elicit any observable mechanical changes in the hydrogels. It was also noted that the samples may not have reached equilibrium at the curtailment of the experiment, and thus H_A would have been incorrectly prescribed, with knock-on effects for the permeability parameters. Furthermore, due to the extreme compliance of the samples, interdigitation of the hydrogels into the porous platen could be a significant source of error. It was also considered that the permeability of the hydrogels was such that it may have approached the permeability of the platen, although later testing of the platen permeability (see **section 6.3.2**) ruled out this particular assumption. However, the main issues were more likely attributable to the insensitivity of the 1 kN load cell, and problems with the device itself. In particular, the increasing amount of perceived friction within the system following repeated use suggested that, although the stress relaxation was typical of poroelastic materials, the recorded peak and

equilibrium stresses were potentially inaccurate, and therefore the calculated biphasic parameters were untrustworthy.

5.4.3 Conclusions from Mechanical Characterisation of Collagen Hydrogels: Part I

The results presented in this chapter suggest that confined compression in combination with biphasic theory is a suitable technique for assessing the mechanical properties of collagen hydrogels. The technique is potentially sensitive to indentify differences between hydrogels of varying collagen concentrations, and thus may also be sensitive to other factors that affect the gel such as matrix remodelling. The primary variables that exhibit this sensitivity are peak stress and permeability, and it is hypothesised that as collagen content (and therefore fibril density) increases, the viscous drag on the fluid flow also increases, resulting in a decrease in the permeability of the hydrogels.

Unfortunately, insensitivities and friction within the system led to inaccuracies in the results, meaning that comparisons of the biphasic parameters with those calculated for other collagenous tissues were not appropriate. It is believed that these issues were mostly attributable to the novel aluminium lid and not the technique itself, although the imprecise measurement system was also partly responsible. Consequently, for all further studies into the mechanical characterisation of collagen hydrogels (see **chapter 6**), alternative methodologies were sought, and the attempt to maintain sterility of samples had its priority lowered. Instead, efforts were focussed on further validation of the technique and theory using a more basic setup and more sensitive equipment.

Chapter 6

Mechanical Characterisation of Collagen Hydrogels: Part II

6 Mechanical Characterisation of Collagen Hydrogels: Part II

6.1 Introduction

Although the data presented in **chapter 5** suggests that confined compression in conjunction with biphasic theory and appropriate constitutive models (Lai & Mow, 1980; Mow *et al*, 1980) may be a suitable technique for mechanical characterisation of hydrogel scaffolds, experimental errors in the results meant that they were not entirely conclusive. It was thought that these issues were most likely a consequence of load cell insensitivities and friction within the system, and an adaptation of the technique was considered necessary to improve the accuracy of recorded measurements. This chapter is concerned with modifications to the confined compression technique and equipment, further validation of the technique, and application of the technique and theory to compare tissue parameters from FPCLs cultured with or without enalapril or captopril.

6.1.1 Adaptation of Confined Compression Technique

While the original concept involved creating a device for compression testing of sterile collagen hydrogels that incorporated a tight fitting aluminium lid to cover standard 24-well tissue culture plates, there were some problems with friction in the system (**section 5.3**), and these issues were believed to be almost entirely attributable to the design of the lid itself. It was therefore decided to no longer incorporate the aluminium lid, and consequently to abandon any attempt at maintaining sterility of samples in favour of improving gel characterisation and further validating the technique and theory for these biomaterials.

Another limitation to the accuracy of previous results was the insensitivity of the employed 1 kN load cell used to the low loads being recorded. Fortunately a more versatile system became available (BOSE ElectroForce® 3200 Test Instrument (BOSE, UK)), allowing for testing with a range of load cells in order to achieve increased sensitivity at an appropriate scale to the forces being measured.

6.1.2 Biphasic Tissue Parameters

As described in **section 5.1.2**, biphasic theory (Mow *et al*, 1980) was chosen to model the stress response of the hydrogels in compression. Although the results in **section 5.3** were inconclusive it was thought that if biphasic theory could be applied successfully using the modified system, meaningful tissue parameters could then be extracted that would allow direct comparison with other biological tissues.

The ability to measure peak and equilibrium stresses following confined compression of hydrogels is useful in that it allows for direct comparison of these parameters between various samples tested in the same way. The equilibrium stress is simply the stress required to deform the matrix by the specified amount (usually 5% strain for the present study). However, the peak stress is a more complex variable, as it is dependent on both experimental and material properties. During the ramp phase, the strain rate ($\partial u/\partial t$) at the boundary between the porous indenter and the surface of the hydrogel is a constant velocity, *v*. Substituting *v* into Eq. (1) (see **section 5.1.2**) gives:

$$\frac{v}{kH_A} = \frac{\partial^2 u}{\partial z^2} \tag{4}$$

where *u* is the displacement of the solid phase in the direction *z*, H_A is the aggregate modulus, and *k* is the hydraulic permeability (*k* and H_A and are discussed in more detail below). Assuming that both *k* and H_A are constant (as for the linear biphasic theory of Mow *et al* (1980)), integrating Eq. (4) with respect to *z* then gives:

$$\frac{vz}{kH_A} + c = \frac{\partial u}{\partial z} \tag{5}$$

where *c* is a constant and $\partial u/\partial z$ is the strain *(ε)*. Since the aggregate modulus is the ratio of stress to strain, it then follows that when the velocity, *v*, is zero:

$$c = \frac{\sigma_{eq}}{H_A} \tag{6}$$

where σ_{eq} is the equilibrium stress. At the boundary, *z* is equal to the height of the sample, *h*, and further modification of Eq. (5) gives the relationship:

$$\sigma_{pk} = \frac{vh}{k} + H_A \varepsilon_{eq} \quad (7)$$

where σ_{pk} is the peak stress. Thus, the peak stress is dependent on the ratio of v to k, and on σ_{eq} (where $\sigma_{eq} = H_A \varepsilon_{eq}$). Peak stress is therefore an amalgam of experimental and material properties: height, ramp speed, hydraulic permeability, aggregate modulus and strain at equilibrium.

It is also desirable to obtain further parameters that provide greater insight into material properties and allow for comparisons with other biological tissues, even where alternative testing methods have been used. The primary variables that exhibit sensitivity to variations in the mechanical properties of biphasic materials are the aggregate modulus *(H_A)* and the zero-strain hydraulic permeability *(k_0)*.

The aggregate modulus *(H_A)* is the stiffness modulus in confined compression, and is related to the Young's modulus, *E*, of the tissue by:

$$H_A = \frac{E(1-\upsilon)}{[(1+\upsilon)(1-2\upsilon)]} \quad (8)$$

where υ is the Poisson's ratio. The ability to measure H_A of a substrate is of great importance as it has been shown that substrate stiffness can significantly influence cell behaviour, affecting factors such as cell adhesion (Discher *et al*, 2005), proliferation (Helary *et al*, 2010), and direction of stem cell lineage specification (Engler *et al*, 2006). Furthermore, the majority of commonly used methods for mechanical characterisation of biological tissues allow for calculation of some form of Young's modulus (**section 1.4.2.1**), meaning that such a parameter could be used to compare tissue characteristics even where different testing methodologies have been used.

Measurement of tissue permeability is also of major importance; for example, in determining a substrate's mechanical properties through effects of hydrostatic pressure and viscous drag to fluid flow. Additionally, while the transport of small metabolites in collagenous tissues is dominated by diffusion (Roberts *et al*, 1996; Urban *et al*, 1978), it is thought that convection may play an important role in the

transport of large essential metabolites such as enzymes and hormones (Ferguson *et al*, 2004; Roberts *et al*, 1996). Indeed, it has been shown that for nucleus pulposus tissue the hydraulic permeability is the limiting factor for convective transport (Riches & McNally, 2005), and thus it appears that hydraulic permeability plays an important role in both the mechanical and metabolic behaviour of collagenous tissues. The zero-strain hydraulic permeability *(k_0)* is a measure of the ability for fluid to flow through a tissue, and a method for estimating k_0 of soft hydrogels is also described herein.

6.1.3 Compression Testing of FPCLs

One of the main motives for developing this novel technique was the requirement to differentiate between scaffolds of varying mechanical properties. Moreover, a major aim of this research was to ascertain whether the inclusion of certain agents could affect the ability for cells to remodel and reshape a collagen-based substrate, leading to changes in its mechanical characteristics.

It was hypothesised that if the technique proved able to differentiate between collagen hydrogels of varying collagen content, then it may also be sensitive to other factors such as matrix remodelling. Consequently, a decision was made to perform confined compression experiments on FPCLs, cultured with or without enalapril or captopril, and over a period that would allow sufficient time for cells within the matrix to have a marked effect on its structural properties. It was hoped that the technique would be sensitive enough to ascertain significant differences in matrix characteristics (if any were present), and in turn to determine whether the presence of the ACE inhibitors does actually affect the mechanical properties of FPCLs over time in culture.

6.2 Materials and Methods

6.2.1 Development of the Technique for Mechanical Characterisation of Collagen Hydrogels

For the purpose of characterising the mechanical properties of collagen-based hydrogels via confined compression, the device and technique detailed in **section 5.2** were modified. Hydrogels were still prepared and tested in 24-well plates and, although the tight fitting aluminium lid was abandoned, the compression platen remained the same, consisting of a narrow stainless steel shaft supporting a circular porous indenter (316L sintered steel mesh) of diameter ~15.9 mm. Instead of using ferrous metal discs and magnets, the platen was simply held rigid in a clamp specifically designed for attachment to the load cell *(Fig. 6.1(b))*. Early testing was performed using a 450 N load cell (BOSE, UK), though for the majority of testing a 10 N load cell (Honeywell, NJ, USA) was employed for greater sensitivity. The linear actuator was controlled via a BOSE ElectroForce® 3200 Test Instrument *(Fig. 6.1(a))* and the accompanying WinTest® software, which was also used for data acquisition for both displacement and load. The technique is intended to enable the direct and indirect determination of the mechanical properties of tissue samples via confined compression and biphasic theory (Lai & Mow, 1980; Mow *et al*, 1980).

All collagen gel samples for compression testing were 16 mm diameter and approximately 5 mm thick (1 ml total volume), and were detached from the sides of the tissue culture wells immediately prior to testing. Preliminary studies were concerned with further defining a protocol for testing hydrogels, and a variety of test conditions were assessed. 0.3% w/v collagen gels were compressed by between 5 and 20%, at 0.025 to 0.05 mm/s, and held for 120 to 300 s.

6.2.2 Estimation of the Permeability of the Porous Platen

To estimate the permeability of the stainless steel porous platen it was submerged fully in dH_2O and subjected to a 4 Hz sinusoidal displacement of 7 mm. The permeability could then be calculated from Eq. (9), which was rearranged from part of the proportionality constant in Darcy's Law that relates the flow rate and fluid physical properties to a pressure gradient applied to a porous media (Darcy, 1856). k is the hydraulic permeability, v is the velocity of the platen, ΔP is the applied pressure difference, and ΔZ is the thickness of the platen.

$$k = v\frac{\Delta Z}{\Delta P} \tag{9}$$

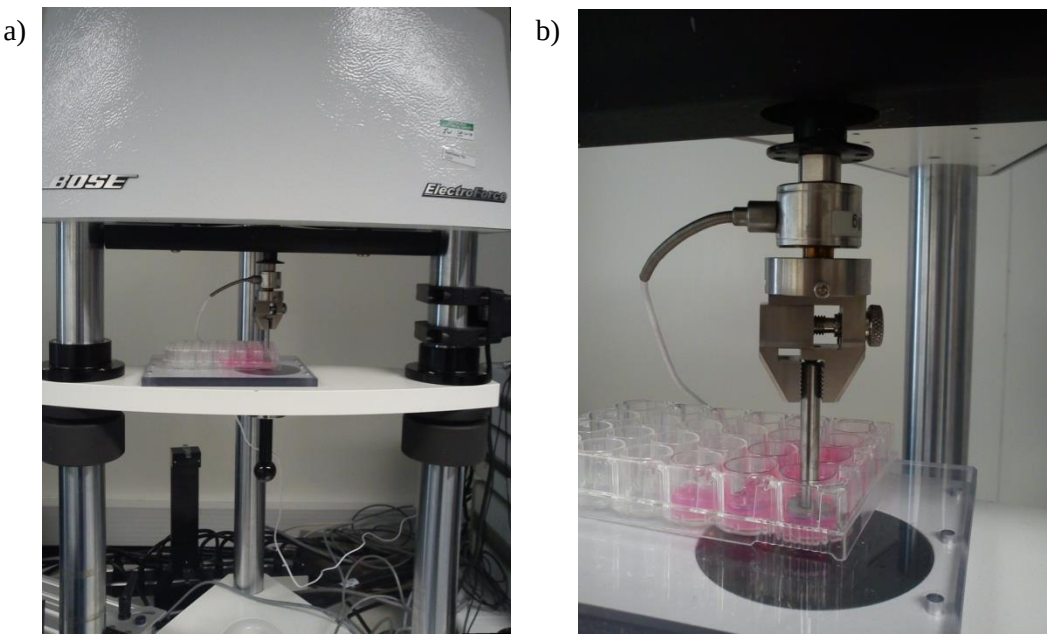


Figure 6.1. a) Photograph of BOSE ElectroForce® 3200 Test Instrument during compression testing of collagen hydrogels. b) Close-up photograph of confined compression setup. Collagen hydrogels in standard 24-well tissue culture plates are compressed with a stainless steel porous indenter attached to a 10 N load cell via a clamp.

6.2.3 Validation of the Technique and Theory for Mechanical Characterisation of Collagen Hydrogels

To validate the theory and technique, collagen gel samples were made at each of three collagen concentrations (0.2%, 0.3% and 0.4%). Gels were detached from the sides of the tissue wells immediately prior to testing, and all excess fluid was removed. The surfaces of the gels were found by lowering the platen until a pre-load of 0.01 N was achieved and then resting the system until the stress response equilibrated, and the sample thickness was deduced from the displacement applied to reach this tare load. Samples were then compressed by 5% at 0.5%/s and held for 300 seconds. Biphasic theory (Lai & Mow, 1980; Mow *et al*, 1980) was fitted to the ramp and hold phases using a Nelder-Mead scheme (Riches, 2011) and tissue parameters, such as H_A and k_0, in addition to the peak and equilibrium stresses, were compared using ANOVA followed by Tukey's test.

6.2.4 Confined Compression Testing of FPCLs

0.3% w/v collagen gels were prepared in 24-well plates at a volume of 1 ml/gel. As required certain collagen gels were prepared with 1 mM of either enalapril or captopril dissolved in their pre-gelled solutions. Special care was taken to ensure that gelation conditions were similar for all samples. 3T3 cells were seeded onto the gels in 1 ml growth medium at a concentration of $2 \times 10^4/cm^2$ and incubated at 37 °C for 3, 6, 9 or 12 days, with fresh medium being applied every 3 days where appropriate. An equal number of samples were prepared without cells and cultured in a similar manner to the FPCLs, with an extra set of cell-free samples also being prepared as a control for testing on the first day of the experiment. Following incubation the 24-well plates were removed and allowed to cool to room temperature before testing commenced. Individual hydrogels were detached from the side of the tissue culture well and all excess fluid was removed immediately prior to testing according to the method detailed in **section 6.2.3**. Tissue parameters obtained over the full range of the experiment were compared by two-way ANOVA followed by Dunnett's test with respect to control samples or Tukey's test with respect to each other, and results from

each individual day or for each individual sample type (control, enalapril, or captopril) were compared by one-way ANOVA followed by Dunnett's test with respect to the relevant control samples or Tukey's test with respect to each other.

6.3 Results

6.3.1 Development of the Technique

Initial compression testing of collagen hydrogels using the setup described in **section 6.2.1** with the 450 N load cell resulted in very uneven stress responses being recorded *(Fig. 6.2)*. However, the stress responses in *Fig. 6.2* are sufficiently smooth to allow a somewhat typical biphasic response to be observed, and also to deduce that a compression rate of 50 µm/s (as was employed for the majority of compression testing in **chapter 5**) is too fast, resulting in a very noisy stress response, particularly around the peak area. A compression rate of 25 µm/s was used for all subsequent compression tests.

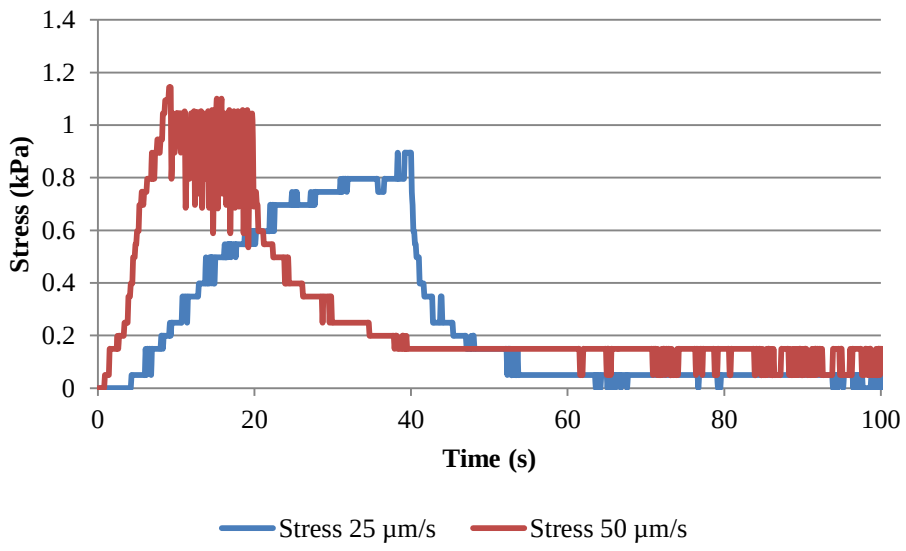


Figure 6.2. Comparison of typical stress responses from confined compression of 0.3% w/v collagen hydrogels, plotted against time. Gels were compressed by 250 µm (~5%) at 25 µm/s or 50 µm/s and held. Results were recorded via a 450 N load cell.

Replacing the 450 N load cell with a 10 N load cell resulted in far smoother stress responses, as seen in *Fig. 6.*3. Although it was initially intended to test the hydrogels submerged in growth medium, typical stress responses from hydrogels tested in this manner demonstrated a staggered series of peaks and troughs during the ramp phase, and negative values following the hold phase (blue line in *Fig. 6.3*). Removing excess fluid before testing resulted in a far smoother ramp phase and positive equilibrium stresses, although it was noted that occasionally the response would appear noisy around the peak when the hydrogels were compressed by 10% (red line in *Fig. 6.3*). Compressing the hydrogels by more than 10% confirmed that stress responses were generally smooth up to around (or just below) 10% strain, and that beyond this point the response would become noisy, with large variations in the response becoming apparent as the strain approached 20% (green line in *Fig 6.3*). For all further compression testing 5% strain was adopted, which consistently allowed for a sufficiently large yet smooth stress response during confined compression, and is appropriate for application of the infinitesimal theory of Mow *et al* (1980).

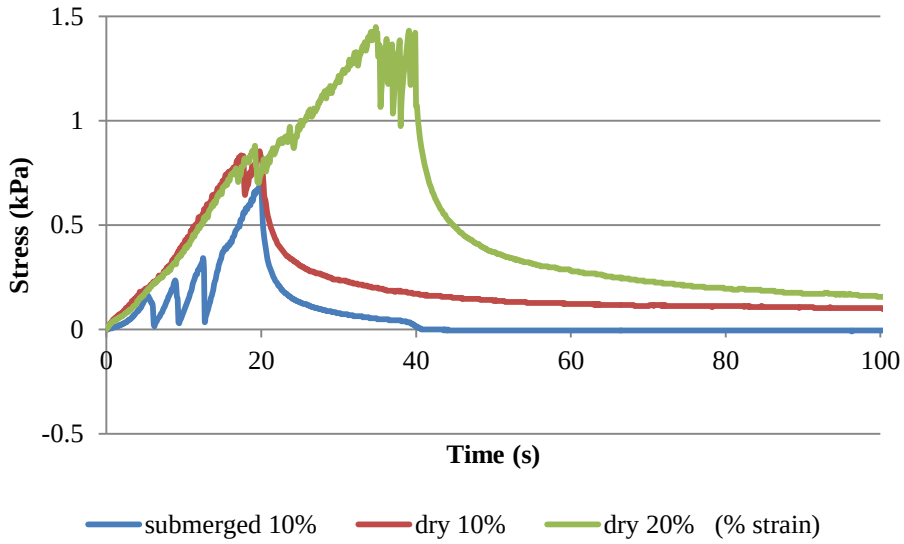


Figure 6.3. Comparison of typical stress responses from confined compression of collagen hydrogels either submerged in growth medium ('submerged') or with the excess fluid removed ('dry'), and subjected to varying amounts of strain, plotted against time. Platens were lowered until a pre-load of 0.01 N was achieved and then held until the stress response equilibrated. Samples were then compressed by 250 µm at 25 µm/s and held.

6.3.2 Permeability of the Porous Platen

Moving the porous platen axially through dH_2O at a speed of 0.028 m/s resulted in a maximum pressure difference of 79.05 N/m^2 (results not shown). As the thickness of the platen was known to be 1.6 mm, its permeability could be calculated using Eq. (9), and was approximated to be 5.95×10^{-7} m^4/Ns.

6.3.3 Validation of Technique and Theory

Compression testing of three different concentrations of collagen hydrogels provided very clear stress responses with minimal noise or discrepancies due to frictional artefacts, and extremely well defined ramps, peaks and hold phases as detailed in

Fig. 6.4. The stress responses in *Fig. 6.4* are highly indicative of biphasic behaviour, and also appear to increase in magnitude relative to collagen content.

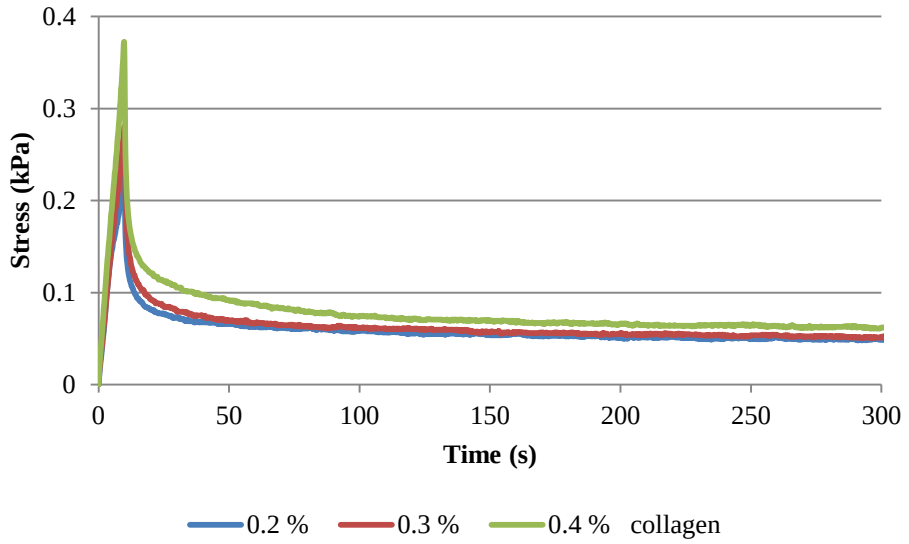


Figure 6.4. Average compressive stresses for hydrogels of three different collagen concentrations in confined compression, plotted against time. Platens were lowered until a pre-load of 0.01 N (0.05 kPa) was achieved and then held until the stress response equilibrated. Samples were then compressed by 5% at 25 µm/s and held for 300 s. Results are mean values, n = 8.

ANOVA of these results confirmed that both peak stresses ($p < 0.001$) and equilibrium stresses ($p = 0.016$) demonstrated significant increases relative to collagen content. Post hoc tests showed that there were significant differences in peak stress between all concentrations of collagen hydrogel tested *(Fig. 6.5(a))*. Post hoc tests on the results for equilibrium stresses demonstrated statistically significant differences between 0.2% and 0.4%, and 0.3% and 0.4% collagen hydrogels, but not between 0.2% and 0.3% hydrogels *(Fig. 6.5(b))*.

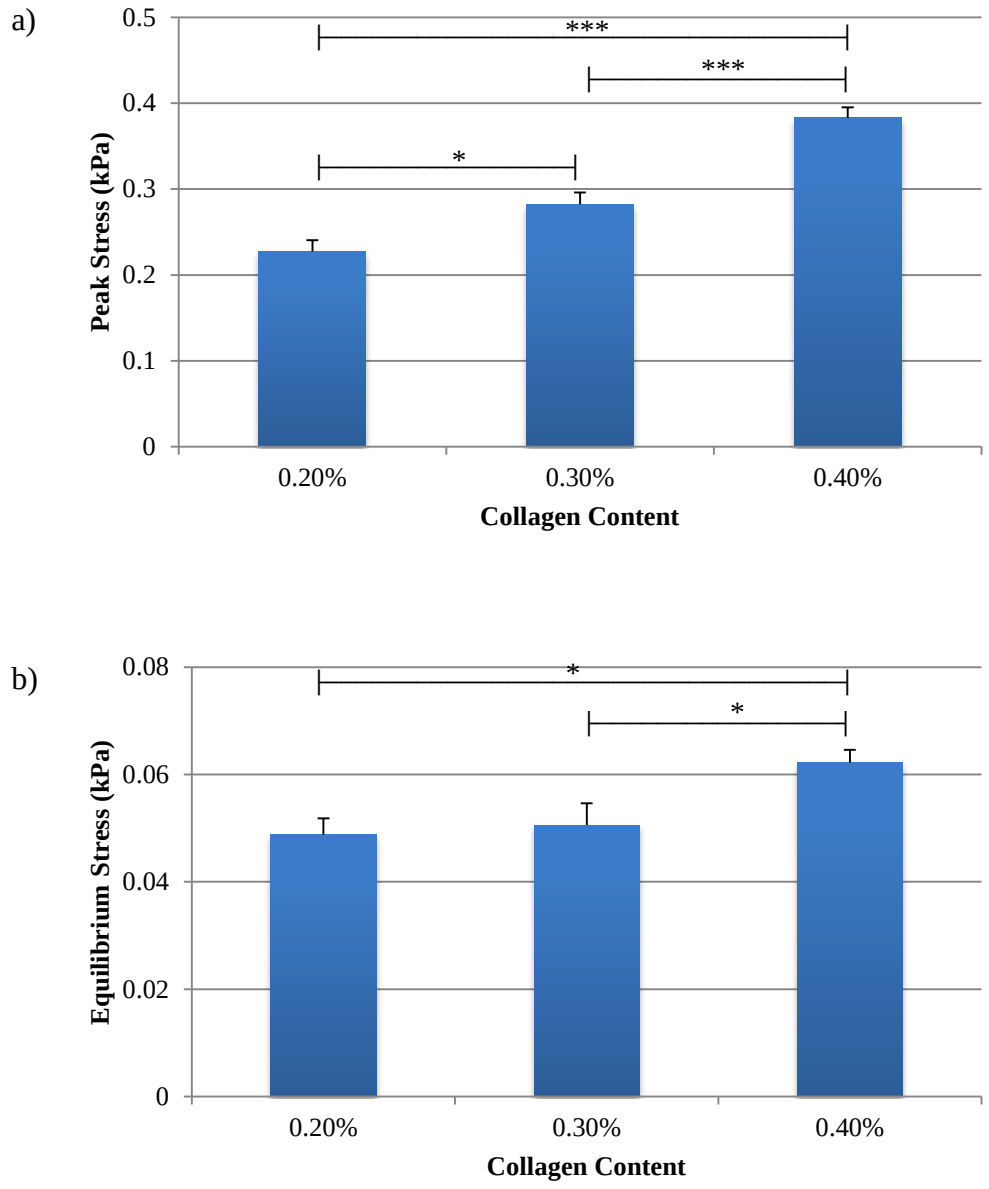


Figure 6.5. Relationship between collagen content and a) peak compressive stress and b) equilibrium compressive stress for hydrogels in confined compression. Results are mean + SEM, n = 8, and were compared by ANOVA followed by Tukey's test. Significant variations: * $p < 0.05$, *** $p < 0.001$, 95% confidence.

The clearly defined ramp and hold phases meant that biphasic theory could be fitted to the entire stress response for each hydrogel in compression. *Fig. 6.6* shows a typical stress response from a collagen hydrogel in confined compression overlaid with the biphasic model, indicating that the model is a good approximation.

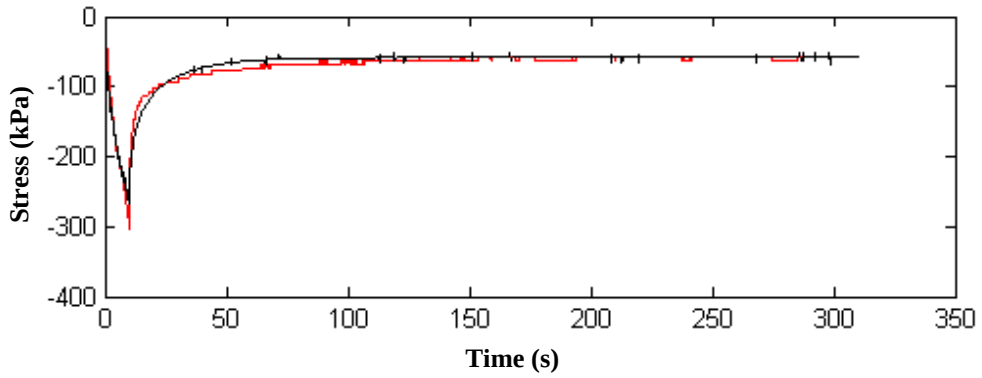


Figure 6.6. Typical stress response from confined compression of a collagen hydrogel (**red** line), overlaid with mathematical model (**black** line). Sample was compressed by 5% at 0.5%/s and held for 300 s. Values are negative because the lines represent the true stress measurements, as opposed to the compressive stress.

ANOVA showed that zero-strain hydraulic permeability varied significantly with respect to collagen content ($p = 0.015$), decreasing with increasing collagen concentration. Post hoc tests demonstrated that the only statistically significant differences ($p = 0.011$) were between the 0.2% and the 0.4% collagen hydrogels *(Fig. 6.7(a)).* ANOVA also demonstrated that aggregate modulus varied significantly with respect to collagen content ($p = 0.008$), increasing relative to collagen concentration, and again post hoc tests revealed significant differences ($p = 0.006$) between only the 0.2% and 0.4% collagen hydrogels *(Fig. 6.7(b)).*

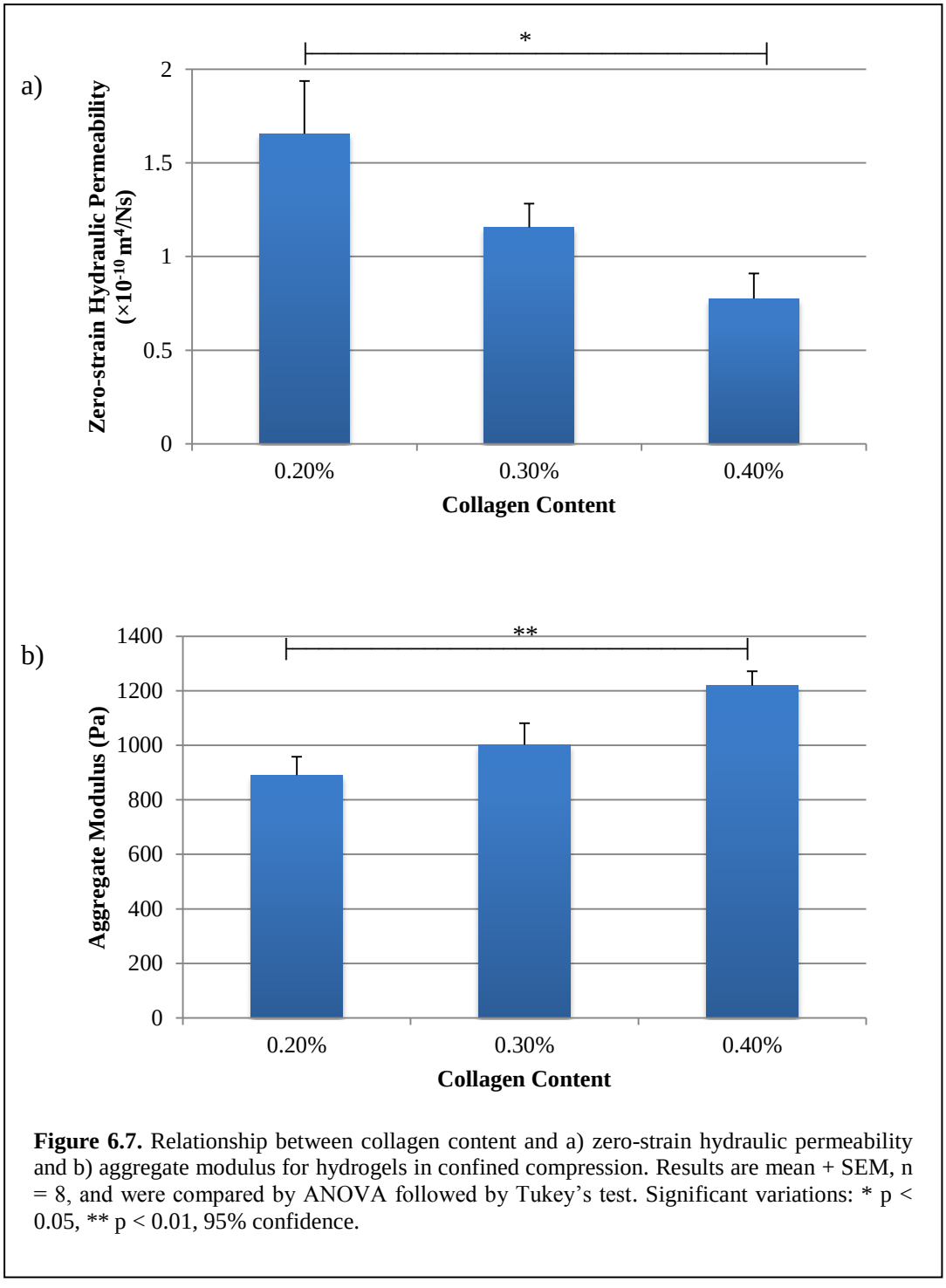


Figure 6.7. Relationship between collagen content and a) zero-strain hydraulic permeability and b) aggregate modulus for hydrogels in confined compression. Results are mean + SEM, n = 8, and were compared by ANOVA followed by Tukey's test. Significant variations: * $p < 0.05$, ** $p < 0.01$, 95% confidence.

6.3.4 Confined Compression Testing of FPCLs

The effect of captopril or enalapril on the mechanical characteristics of collagen hydrogels was measured in cell-free hydrogels and in FPCLs that had been in culture for 3, 6, 9 or 12 days, and in cell-free hydrogels that were tested on the first day of the experiment (day 0). For the cell-free samples, a one-way ANOVA followed by Dunnett's test of peak stresses from each day suggested significant increases relative to control samples at day 6 ($p < 0.01$), and days 9 and 12 ($p < 0.05$) for captopril treated samples, and day 12 ($p < 0.001$) for enalapril treated samples *(Fig. 6.8(a))*. Two-way ANOVA followed by Dunnett's test over the entire range of results for cell-free hydrogels further demonstrated that there were significant variations in peak stresses between the control and both enalapril ($p = 0.016$) and captopril ($p = 0.001$) treated samples. Comparing the results from cell-free collagen hydrogels also demonstrated that peak stresses for all samples decreased with respect to time in culture, except at day 12 where there was an increase in peak stress for the enalapril treated samples, and this was also confirmed by one-way ANOVA ($p < 0.001$ for control, enalapril; $p < 0.01$ for captopril).

One-way ANOVA followed by Dunnett's test of the FPCL measurements from each individual day showed significantly larger peak stresses (compared to controls) for samples treated with either ACE inhibitor at days 6 and 9 ($p < 0.001$), and day 12 ($p < 0.01$), although a significantly lower peak stress ($p < 0.05$) was also measured on day 3 for the captopril treated samples *(Fig. 6.8(b))*. Two-way ANOVA followed by Dunnett's test confirmed significant variations in peak stresses between the control samples and samples treated with either ACE inhibitor ($p < 0.001$), and Tukey's test suggested no significant variation between the enalapril and captopril treated samples. Further analysis of the FPCL results showed no significant variation in the measured peak stresses of the control samples over the course of the experiment, while there were significantly larger peak stresses ($p < 0.001$) at days 6, 9, and 12 for both enalapril and captopril treated samples when compared with their respective day 3 measurements or with their respective day 0 cell-free measurements.

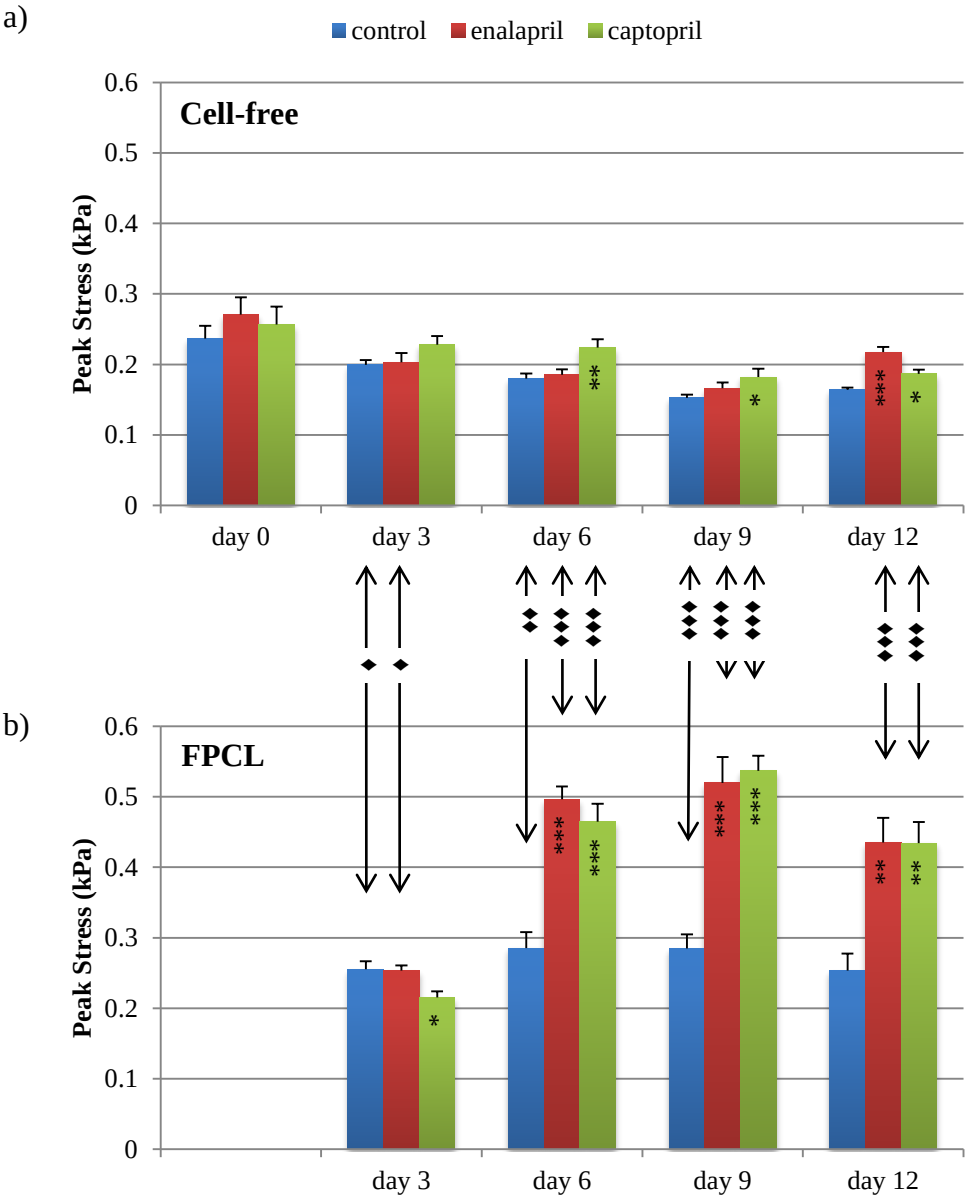


Figure 6.8. Relationship between peak compressive stress in confined compression and time in culture for a) cell-free collagen hydrogels and b) FPCLs. Results are mean + SEM, n = 8, and were compared with controls by two-way ANOVA followed by Dunnett's test over the full range of results, and one-way ANOVA followed by Dunnett's test at each day. ANOVA followed by Tukey's test was used to compare cell-free samples with FPCLS at each day. Significant variations relative to control sample on same day: *p < 0.05, ** p < 0.01, *** p < 0.001. Significant variations between FPCLs and equivalent cell-free samples: ♦p < 0.05, ♦♦p < 0.01, ♦♦♦p < 0.001. 95% confidence.

It was evident that over the course of the experiment the measured peak stresses were generally significantly larger for all the FPCLs than for their equivalent cell-free samples. These differences were relatively small for the control samples, but much more pronounced for the ACE inhibitors treated samples, with enalapril and captopril treated FPCLs demonstrating peak stresses in the region of twice those of their equivalent cell-free samples on days 6, 9 and 12 *(Fig. 6.8)*.

There were no significant differences between the equilibrium stresses of the cell-free samples at each individual day *(Fig. 6.9(a))*. There were however significantly larger equilibrium stresses for enalapril ($p < 0.001$) and captopril ($p < 0.05$) treated FPCLs compared with control FPCLs on day 9 *(Fig. 6.9(b))*, as confirmed by one-way ANOVA followed by Dunett's test of the results from each day. Two-way ANOVA followed by Dunnet's test of both complete sets of results confirmed no significant differences in the results for cell-free hydrogels ($p > 0.9$), but large significant differences between ACE inhibitor treated samples and the control samples ($p < 0.001$) for the FPCLs.

Further analysis showed that the control and enalapril treated cell-free samples demonstrated significantly larger equilibrium stresses at days 3 and 12 ($p < 0.05$), compared with day 0. Equilibrium stresses for the captopril treated cell-free samples also appeared to increase over the course of the experiment *(Fig. 6.9(a))*, though these increases were not significantly different relative to time in culture. In contrast, the equilibrium stresses of the control FPCLs appeared to remain relatively constant over the course of the experiment and did not vary significantly *(Fig. 6.9(b))*, even when compared with the measurements for the cell-free samples on day 0. The equilibrium stresses of the ACE inhibitor treated FPCLs also did not vary significantly with respect to time in culture, though measurements from enalapril treated FPCLs were significantly larger on days 6, 9, and 12 than for the equivalent cell-free samples on day 0. Measured equilibrium stresses for the control FPCLs were considerably lower than for the equivalent cell-free samples at each time point, though results were not statistically significant. In contrast, equilibrium stresses for the enalapril and captopril treated FPCLs were generally much more similar to those measured for their equivalent cell-free samples *(Fig. 6.9)*.

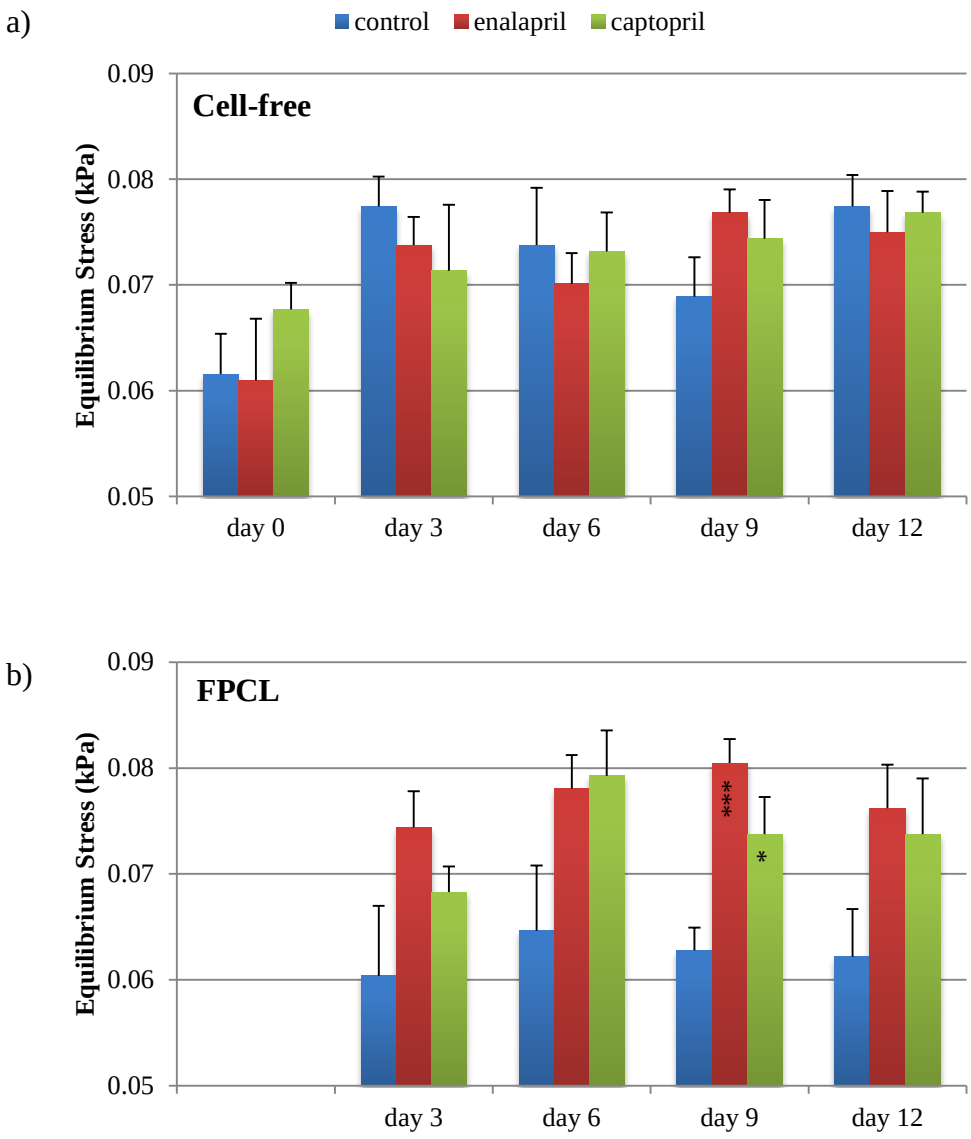


Figure 6.9. Relationship between equilibrium compressive stress in confined compression and time in culture for a) cell-free collagen hydrogels and b) FPCLs. Results are mean + SEM, n = 8, and were compared with controls by two-way ANOVA followed by Dunnett's test over the full range of results, and one-way ANOVA followed by Dunnett's test at each day. ANOVA followed by Tukey's test was used to compare cell-free samples with FPCLS at each day. Significant variations relative to control sample on same day: *p < 0.05, *** p < 0.001, 95% confidence.

The captopril treated FPCLs demonstrated a significantly higher zero-strain hydraulic permeability (k_0) than the control samples on day 3 *(Fig. 6.10(b))*, though there were no other significant variations in k_0 at each individual day between the ACE inhibitor treated samples and the control samples for either the cell-free samples or the FPCLs. Two-way ANOVA showed that k_0 did not vary significantly with respect to the presence or absence of ACE inhibitors over the course of the experiment for either the cell-free samples or the FPCLs.

The zero-strain hydraulic permeability for all cell-free samples appeared to increase steadily until day 9 then decrease on day 12 *(Fig. 6.10(a))*, whereas for the FPCLs k_0 appeared to increase on day 3 for the enalapril and captopril treated samples and then noticeably decrease on day 6, decreasing further on days 9 and 12 *(Fig. 6.10(b))*. *Fig 6.10(b)* also suggests that k_0 of the control FPCLs steadily decreased until day 12, when there was a perceived increase but also a large variance. Further analysis showed significant increases in k_0 for the enalapril ($p < 0.001$) treated cell-free samples on days 9 and 12 when compared with their respective day 0 measurements. k_0 of the control FPCLs did not vary significantly over the course of the experiment, whereas the enalapril and captopril treated FPCLs had a significantly lower k_0 on days 6, 9 and 12 than on day 3. Calculated values of k_0 for the FPCLs were also generally lower than for their equivalent cell-free samples from day 6, with significant variations for the control and enalapril treated samples on day 6, and for all samples on days 9 and 12 *(Fig. 6.10)*.

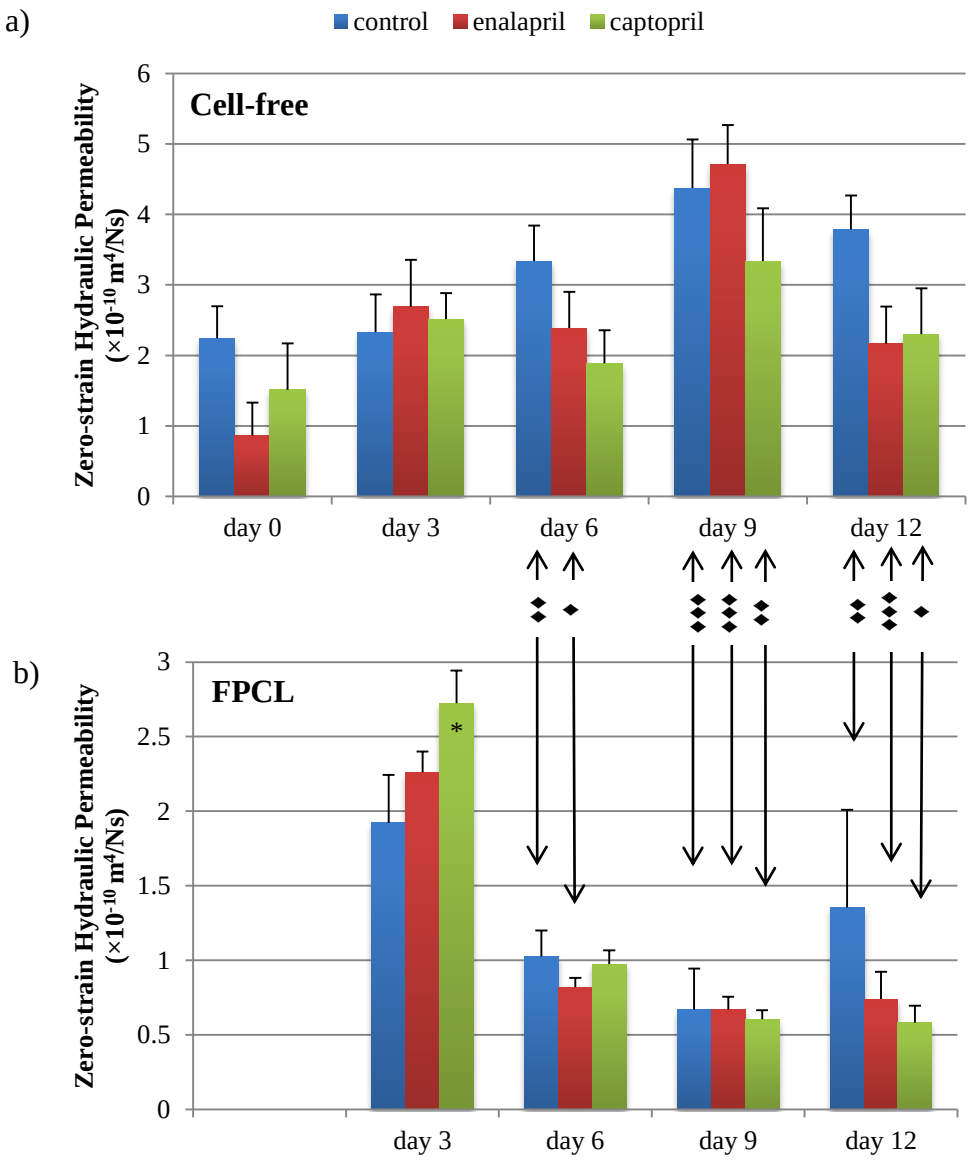


Figure 6.10. Relationship between zero-strain hydraulic permeability (k_0) and time in culture for a) cell-free collagen hydrogels and b) FPCLs. Results are mean + SEM, n = 8, and were compared with controls by two-way ANOVA followed by Dunnett's test over the full range of results, and one-way ANOVA followed by Dunnett's test at each day. ANOVA followed by Tukey's test was used to compare cell-free samples with FPCLS at each day. Significant variations relative to control sample on same day: *p < 0.05. Significant variations between FPCLs and equivalent cell-free samples ♦p < 0.05, ♦♦p < 0.01, ♦♦♦p < 0.001. 95% confidence.

One-way ANOVA followed by Dunnett's test of the results from each specific day confirmed significantly larger aggregate modulus (H_A) on day 9 for enalapril ($p < 0.001$) and captopril ($p = 0.017$) treated FPCLs compared with the control FPCLs *(Fig. 6.11(b))*, and no significant variations between the ACE inhibitor treated cell-free samples and their relative controls *(Fig 6.11(a))*. Two-way ANOVA followed by Dunnett's test over the entire range of results confirmed significant differences in H_A between the control FPCLs and both the captopril ($p = 0.001$) and enalapril ($p < 0.001$) treated FPCLs, while there was no significant variation in the results for the cell-free hydrogels.

H_A of the cell-free hydrogels appeared to increase for all samples following the first day of testing *(Fig. 6.11(a))*, though additional analysis (one-way ANOVA) showed that only the enalapril treated samples demonstrated significant variation in H_A over the course of the experiment, with significantly larger H_A on day 3 ($p = 0.042$), and on days 9 and 12 ($p = 0.008$), compared to results from day 0. H_A of the FPCL samples did not vary significantly over the course of the experiment. However, *Fig 6.11(b)* suggests that while the control FPCLs did maintain a relatively constant H_A throughout the experiment, both captopril and enalapril treated FPCLs displayed marked increases in H_A, with peaks at days 6 and 9 for captopril and enalapril respectively. Values of H_A for the control FPCLs were also noted to be considerably lower than for the equivalent cell-free samples at each time point, though results were not statistically significant. In contrast, calculated values of H_A for the enalapril and captopril treated FPCLs were generally very similar to their equivalent cell-free samples.

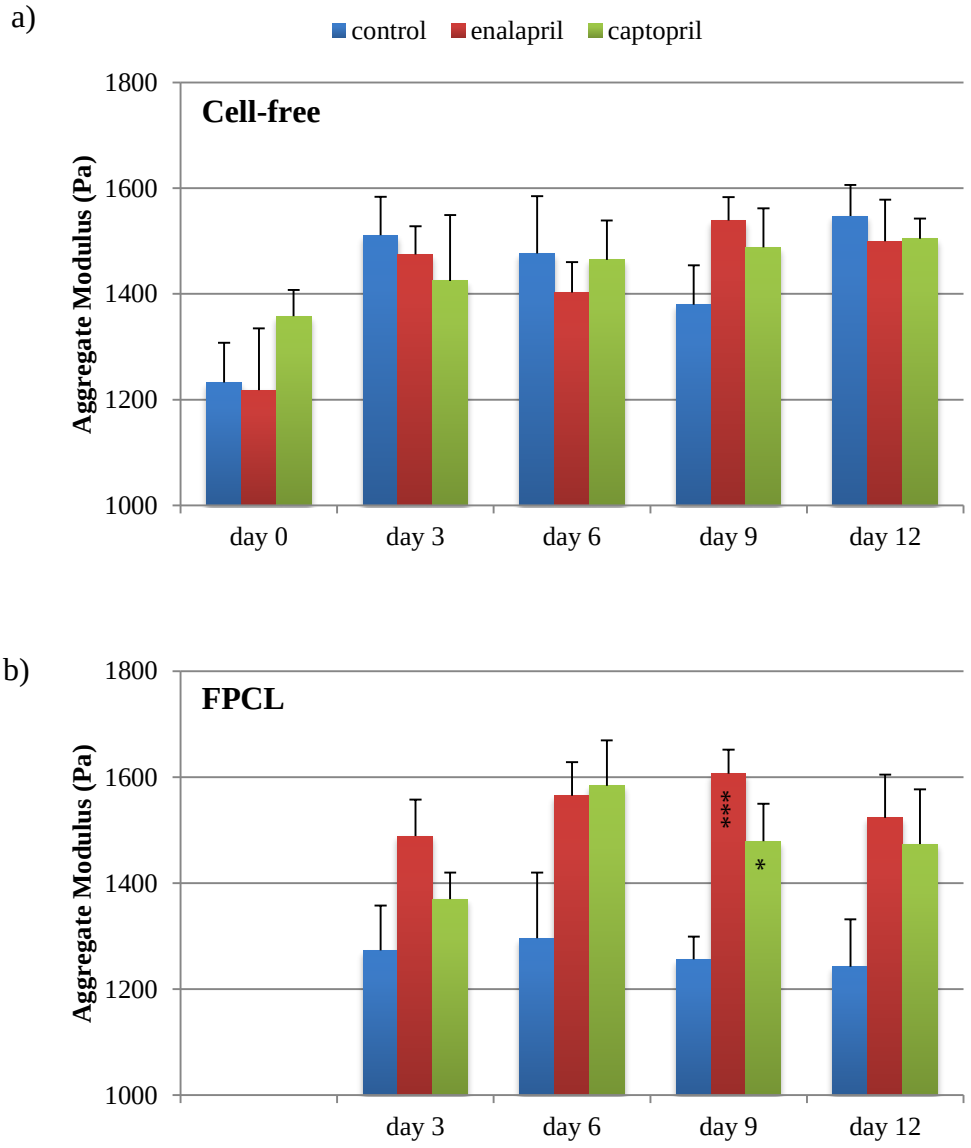


Figure 6.11. Relationship between aggregate modulus (H_A) and time in culture for a) cell-free collagen hydrogels and b) FPCLs. Results are mean + SEM, n = 8, and were compared with controls by two-way ANOVA followed by Dunnett's test over the full range of results, and one-way ANOVA followed by Dunnett's test at each day. ANOVA followed by Tukey's test was used to compare cell-free samples with FPCLS at each day. Significant variations relative to control sample on same day: *$p < 0.05$, *** $p < 0.001$, 95% confidence.

6.4 Discussion

The results and analysis presented in **chapter 5** suggest that confined compression and biphasic theory (Lai & Mow, 1980; Mow *et al*, 1980) may be a suitable technique to ascertain differences in the mechanical characteristics of collagen hydrogel scaffolds. However, inconclusive results attributable to problems with the equipment meant that improvements to the technique were considered necessary.

6.4.1 Adaptation of Confined Compression Technique

In order to improve the accuracy of measurements, some major modifications were made to the technique described in **section 5.2**. One of the main problems with the original setup had been friction in the system causing discrepancies in the results and, more specifically, large instantaneous jumps in the recorded stresses during the compressive phase of testing. It was assumed that the majority of this friction was attributable to the aluminium lid and the linear bearings that were employed to guide the porous indenters. Consequently, a decision was made to abandon the lid altogether, and to simply attach the porous indenters to the load cell via a clamp instead of the magnets used previously. Although this major simplification to the confined compression setup meant that samples would no longer remain sterile during testing, it was considered of greater importance to improve the accuracy and validity of the technique. Another major modification to the setup was the use of a BOSE ElectroForce® 3200 Test Instrument, which in terms of capabilities is more versatile and intuitive than the Zwick/Roell system used previously (see **section 5.2**), and also allowed for a variety of load cells to be attached, ultimately resulting in increased sensitivity.

Compression testing using the modified setup and a 450 N load cell suggested that the sensitivity of the system still required improvement, as stress responses appeared rough and angular due to the lack of resolution in measurement. However, despite the lack of precision, these early tests suggested that the strain rate that had been adopted for the majority of testing in **chapter 5** was in fact too high, and did not

provide a steady response. A lower strain rate was therefore adopted for all further testing, and even tests with the 450 N load cell demonstrated a typical biphasic response of sufficient magnitude for subsequent analysis.

Replacing the 450 N load cell with a 10 N load cell resulted in far smoother stress responses and clearly showed that there were no longer large jumps in the responses during the compressive phase, suggesting that, as hypothesised, this particular issue could be entirely attributed to problems with the design of the aluminium lid used previously. Having achieved typical biphasic stress recordings without obvious experimental errors using the modified setup, checks were again made into the effects of testing hydrogels in fluid, or with excess fluid removed. Although the compression testing in **chapter 5** was predominantly performed on hydrogels fully submerged in growth medium, typical stress responses from submerged hydrogels using the new setup showed ramp phases consisting of a staggered series of peaks and troughs, and negative values following the hold phase. The reason for these irregularities may be attributed to fluid being trapped beneath the floating hydrogels while the platen is being lowered prior to testing, and then escaping around the periphery of the sample (as opposed to through it) as more pressure is applied during further compression. It proved extremely difficult to ensure that no fluid remained trapped under the hydrogels prior to testing, and consequently all further tests were performed with any excess fluid removed immediately prior to testing.

It has been suggested that the stress response of collagen is linear up to ~10% strain (Barocas *et al*, 1995). Nonetheless, a decision was made to investigate the strain range 0-20% using the new setup. The results demonstrated that the stress response from the collagen hydrogels was indeed relatively linear and smooth up to ~10% strain, at which point there was a considerable increase in noise which continued as the strain was increased further. As strain approached 20%, large variations in the response became apparent, suggesting that at these larger strains the hydrogels lose some of their elasticity, and may be suffering from permanent deformation and probably interdigitation of the gel into the porous platen due their extreme compliance. Since it was necessary to connect the load cell in series with the linear actuator, it is also possible that the load cell picked up small vibrations from the

linear motor. If these vibrations were close to the resonant frequency of a certain stiffness of hydrogel, they would be amplified at this stiffness. Placing the load cell beneath the 24-well plate would avoid the influence of these vibrations, but was not practicable in this case. In order to ensure the gel and boundary conditions remained consistent with the mathematical model a compressive strain of 5% was adopted for all further testing.

6.4.2 Validation of the Technique and Theory for Mechanical Characterisation of Collagen Hydrogels

In order to validate the technique and theory, compression tests were again performed on collagen hydrogels of three different collagen concentrations. Although the results presented in **section 5.3** were mostly inconclusive, it was hypothesised that, due to the modified technique implying far greater sensitivity and accuracy of results than in previous experiments, the extracted parameters would be more likely to vary with collagen content. Indeed, both peak and equilibrium stresses demonstrated significant increases relative to collagen content, suggesting that these parameters could be used successfully to characterise mechanical differences in hydrogel properties. It is also interesting to note that the measured peak stresses were markedly lower than those from similar tests using the previous methodology (see **section 5.3**), further suggesting that the magnitude of recorded measurements was no longer as susceptible to the additive effects of friction.

The ramp and hold phases during compression were highly typical of poroelastic materials, and consequently biphasic theory was fitted to the whole response. It was shown that the biphasic model was a good approximation for the individual stress responses, which in turn increases the likelihood of accurate tissue parameter calculations. It has been shown that increasing the collagen content in hydrogels results in a greater fibril density while maintaining a relatively constant fibril diameter (Roeder *et al*, 2002). It was therefore hypothesised that as collagen content increases, the zero-strain hydraulic permeability (k_0) of a substrate should decrease, because a larger collagen content, and hence fibril density, would be expected to

increase the viscous drag on the fluid flow. As expected k_0 did decrease with increasing collagen content, showing that the technique can be used to differentiate between hydrogels of varying permeabilities. It has also been demonstrated that the stiffness of collagen hydrogels in tension increases relative to collagen content (Krishnan *et al*, 2004; Roeder *et al*, 2002), and consequently it was assumed that a larger fibril density would also translate to an increase in matrix stiffness in compression. This hypothesis was again confirmed through experimentation and subsequent analysis, where results demonstrated significant increases in aggregate modulus (H_A) relative to collagen content in hydrogels.

Characterisation Method	**Property Measured**	**Approximate Values Obtained**	**Authors**
Extensometry	Young's modulus	40 kPa	(Osborne *et al*, 1998)
Extensometry	Incremental modulus	90 kPa	(Saddiq *et al*, 2009)
Rheometry with confined compression (creep)	Aggregate modulus	6.3 kPa	(Knapp *et al*, 1997)
Rheometry with confined compression (ramp)	Aggregate modulus	0.32 kPa	(Knapp *et al*, 1997)
Confined compression with biphasic theory	Aggregate modulus	1 kPa	*Present study*

Table 6.1: Approximate modulus values for 0.3% w/v collagen type I hydrogels found via a variety of experimental techniques.

Comparing the aggregate modulus values obtained in the present study with modulus values obtained by different means for similar scaffolds *(Table 6.1)* demonstrates that the chosen method of characterisation can greatly influence the results. The values in *Table 6.1* suggest that collagen type I is much more stiff in tension than it is in compression. This effect may be because the collagen fibrils buckle relatively easily when compressed, despite exhibiting high strength in tension, and therefore results from extensometry testing cannot easily be compared with those from compressive testing. Where rheometry was used in combination with confined compression the results were more similar to those obtained in the present study, although Knapp *et al* (1997) demonstrated that even minor differences in the characterisation technique used can lead to relatively large differences in the calculated mechanical properties *(Table 6.1)*.

It is also of interest to note how the material properties obtained here for collagen hydrogels compare with those calculated for other biological tissues where similar testing methods and theory have been applied. *Table 6.2* shows that other collagenous tissues (specifically cartilaginous tissues) have an aggregate modulus (stiffness) several hundred times greater than that of 0.3% collagen hydrogels, while the hydraulic permeabilities of these tissues are in the region of 10^5 times lower than those calculated for the hydrogels. These large differences in the mechanical properties between collagen hydrogels and other collagenous tissues are presumably due to the much lower fibril density and lower degree of cross-linking in the hydrogels.

Aggregate Modulus (kPa)	**Hydraulic Permeability (× 10^{-15} m^4/Ns)**	**Tissue**	**Species**	**Authors**
400 ± 140	2.7 ± 1.5	Articular cartilage	Bovine	(Ateshian *et al*, 1997)
310 ± 40	0.67 ± 0.09	Nucleus Pulposus	Bovine	(Périé *et al*, 2005)
740 ± 130	0.23 ± 0.19	Annulus Fibrosis	Bovine	(Périé *et al*, 2005)
560 ± 210	0.18 ± 0.07	Annulus Fibrosis	Human	(Iatridis *et al*, 1998)
1 ± 0.08	0.28 ± 0.01 (× 10^5)	0.3% Collagen Hydrogel	Rat	*Present study*

Table 6.2: Comparison of material properties found via confined compression and biphasic theory for collagenous tissues.

The results presented in this section suggest that confined compression, together with biphasic theory, is a suitable technique for assessing the mechanical properties of collagen-based scaffolds. Material properties and test-dependent experimental stress values exhibited sensitivity to collagen content. Therefore the technique is clearly able to discriminate between hydrogels with small differences in collagen concentration, and potentially may be sensitive to factors that affect matrix remodelling.

The technique itself appears to have several distinct advantages over other commonly used methods for hydrogel characterisation (**section 1.4.2.1**). Most notably, confined compression of samples together with the application of biphasic theory allows for characterisation of the time-dependent properties of the materials, whereas many other techniques simply characterise rubber elastic properties (for a review see Anseth *et al* (1996)), which would appear insufficient as soft hydrated tissues are

fundamentally time-dependent in their nature. Where alternative techniques have been used to measure viscoelasticity in tissues (for example Wagenseil *et al* (2003)), they are still often limited in the range of parameters that can be extracted, and the extracted parameters are not easily attributable to a physical structure. In contrast, the method presented in this study also allows for estimation of the hydraulic permeability of the hydrogels which, in addition to contributing to mechanical properties, is known to significantly influence the convective and diffusional properties of solutes through tissue (Pal *et al*, 2009; Riches & McNally, 2005). While unconfined compression of samples can also be used for estimating tissue permeabilities (for example Soulhat *et al* (1999)), such an approach was not considered for this study as hydrogels of low collagen content can be so compliant that they must be constrained laterally to prevent significant outward deformation, even in the absence of any external forces other than those attributable to gravity.

Another advantage of the technique presented herein is that testing can be performed in standard tissue culture plates with a minimum of disruption to samples prior to testing. Furthermore, although not achieved in the present study, it is possible that with some further modifications the technique could be used to test samples whilst maintaining sterility, which would allow for repeated testing of the same samples at various time points. However, such an approach would also require careful selection of controls, especially when testing cell-seeded constructs, as cyclic loading of cells in tissue constructs has been shown to influence protease production (Prajapati *et al*, 2000), and even a single application has been shown to accelerate *in vitro* tissue formation and consequently affect tissue mechanical properties (Waldman *et al*, 2006). Nevertheless, the ability to maintain sterility for mechanical testing of tissue samples is of particular importance for time-dependent studies, and the majority of commonly used methods for mechanical characterisation of hydrogels do not allow for sterile testing or are extremely troublesome and unlikely to allow for high throughput of samples (see **section 1.4.2.1**). Therefore, it appears that with appropriate modifications the confined compression technique presented in this study could be useful for measuring variations in the mechanical properties of tissue constructs during long-term studies, although there would be a limitation in that cell densities sufficient to contract detached scaffolds laterally would result in the

scaffolds no longer being confined, leading to discrepancies in the measured and calculated parameters.

6.4.3 Confined Compression Testing of FPCLs

Collagen in the body undergoes a constant process of remodelling and reshaping. This process was for many years poorly understood, and early studies of collagen turnover *in vivo* suggested that in adult animal tissues collagen was in fact a relatively inactive protein, and that where it was synthesised or degraded the processes happened extremely slowly (Neuberger *et al*, 1951; Neuberger & Slack, 1953). However, as techniques to measure collagen turnover *in vivo* have improved it has become apparent that collagen is a much more active protein than previously thought, and also that turnover rates are highly variable between different tissues (Laurent, 1987; Nissen *et al*, 1978; Rucklidge *et al*, 1992). Of particular interest to the present study is the fate of implanted collagen, and McPherson *et al* (1986) demonstrated that collagen injected into the guinea pig dermis had a half-life of only 4 days. It should be noted that a similarly high rate of degradation would probably not be expected in humans (presumably due to a higher metabolic activity of guinea pig dermis) as it has also been indicated that significant amounts of collagen implanted in the human dermis remained 6 months post implantation (McPherson *et al*, 1986). Nonetheless, high rates of substrate degradation are unlikely to be supported by sufficient synthesis of new collagen to maintain the structure of the scaffold. Consequently, there is a requirement for methods to assess the mechanical effects of cell ingrowth into prepared scaffolds that have been designed for implantation or tissue replacement strategies.

As fibroblasts are one of the most active cell types in terms of ECM remodelling, they are often used to assess the effects of cell ingrowth into collagen hydrogels. The majority of reports are concerned with floating (i.e. unconstrained) FPCLs, where cells mediate extensive contraction of the substrate, leading to an increased collagen density and, consequently, increased mechanical strength (for example, (Feng *et al*, 2003a; Feng *et al*, 2003b; Huang *et al*, 1993; Wakatsuki *et al*, 2000)). However, in

the present study FPCLs were not allowed to float freely and were left anchored to the sides of the culture dishes, allowing minimal cell-mediated contraction to take place. Such an approach seems more appropriate for examining tissue replacement strategies because scaffolds that are designed to be a particular shape and size will become anchored to other biological structures in the body following implantation, and therefore contraction of the matrix will be restricted.

Having already confirmed that confined compression together with biphasic theory is a suitable and effective technique for the mechanical characterisation of collagen hydrogels, it was then used to compare the effects of enalapril and captopril on the mechanical properties of FPCLs during time in culture. All collagen hydrogels were tested using the aforementioned confined compression technique (see **section 6.2.4**), and material properties and test-dependent experimental stress values were compared for FPCLs in the presence or absence of dissolved enalapril or captopril, and also for a similar set of control hydrogels that were not seeded with cells. EDTA was not used as a positive control for collagenase inhibition in this instance because it is known to be toxic to cells in culture following prolonged exposure (Amaral *et al*, 2007; Ballal *et al*, 2009), and it can also disrupt cell adhesion to the ECM leading to cell lysis (Gilbert *et al*, 2006).

It has previously been shown that cells cultured in anchored FPCLs degraded the matrix more quickly than they were able to synthesise new structural proteins, resulting in a loss of matrix stiffness (Saddiq *et al*, 2009), and also that enalapril and captopril are able to inhibit the degradative action of collagenase at non-toxic concentrations (see **chapter 4**). Therefore, it was hypothesised that if the ACE inhibitors had any influence on the mechanical characteristics of the FPCLs, it would most likely be to reduce weakening of the matrices over time. It was thought that the ACE inhibitors may even contribute to strengthening of the matrices by inhibiting collagenolytic breakdown whilst still allowing deposition of new ECM materials, although this was considered unlikely since the omission of ascorbic acid from cell culture is said to result in minimal new collagen synthesis (Chen & Raghunath, 2009).

It was clear from analysis of the test-dependent stress values and the calculated values for H_A that enalapril and captopril did indeed contribute to variations in the mechanical properties of both FPCLs and cell-free hydrogels, though the effect was far more prominent for the FPCLs. However, before substantial conclusions could be drawn it was important to be aware of any effect that the cells themselves may be having on the time-dependent mechanical properties of the substrates. The presence of a cell layer would not be expected to have an appreciable effect on the bulk properties of the matrix, but it could have more localised effects. In the present study the cells would have been most prevalent at the interface where the matrix and the porous platen met, since they had initially been seeded on top of the hydrogels. Therefore, as the cells grew to confluence, it is possible that they would have an effect on the ability for fluid to flow at this interface, which could in turn lead to variations in the measured and calculated time-dependent properties of the hydrogels. Such an effect would be most noticeable as variations in the calculated values for k_0, since changes in permeability at the scaffold's surface cannot be easily distinguished from changes in the overall permeability of the material when using compressive techniques for characterisation. On days 6, 9, and 12 of the experiment, zero-strain hydraulic permeabilities of all the FPCLs were significantly lower than for the cell-free hydrogels, suggesting that the proliferation of the cell layer did indeed result in a decrease in the calculated hydraulic permeability. The fact that this perceived reduction in k_0 only becomes apparent after 6 days in culture is probably due to the time required for the cells to reach a sufficient level of confluence to have a notable effect on the hydraulic permeability at the interface.

Values for k_0 were previously shown to vary with respect to collagen content (**section 6.3.3**), but results from experiments with FPCLs were less conclusive, and for the most part were not indicative of significant variation in k_0 between the control and ACE inhibitor treated samples. It had been speculated that the permeability of the hydrogels was such that it may have approached the permeability of the platen, leading to discrepancies in the results, although analysis of the platen revealed that it was in fact in the region of 10^3 times more permeable than the hydrogels (**section 6.3.2**) and therefore unlikely to be influential in this way. It is also possible that a significant source of error could be attributed to the extreme compliance of the

samples leading to interdigitation of the hydrogels into the porous platen. Another point of note is that the values obtained for k_0 are a consequence of the application of biphasic theory, which involves fitting an equation in a multi-parameter space, and whilst the method may find a numerically optimum set of parameters, they are not necessarily the true parameters of the sample (Riches, 2011). Nonetheless, it appears most likely that the variations in k_0 between the samples over the course of the experiment were simply not large enough to be considered statistically significant, suggesting that the presence of enalapril or captopril did not sufficiently affect the k_0 of FPCLs during culture under these conditions so as to be distinguishable from effects imparted by the proliferating cell layers.

There were also some differences between the measured peak stresses of the FPCLs and the cell-free hydrogels, and even the control FPCLs demonstrated significantly larger peak stresses than the equivalent cell-free hydrogels on days 3, 6 and 9. These results suggest that the mere presence of a cell layer can also have an effect on the measurement of peak stress, which is perhaps to be expected since the cell layer appears to affect k_0, which is directly related to the peak stress during confined compression (see Eq. 7, **section 6.1.2**). However, the presence of the cell layer would not be expected to have a similar influence on the values of equilibrium stress or H_A, since these parameters are not affected by viscous drag to fluid flow, and so any variations in these parameters can be more easily attributed to changes in the bulk material properties.

It is important to note that peak stress is also related to the stiffness of the substrate, and given that the differences in peak stresses between the FPCLs and the cell-free hydrogels were so much greater for the enalapril and captopril treated samples, there is clearly a larger net positive effect on peak stress attributable to the combination of cells and ACE inhibitors than to the presence of the cell layer alone, which may be indicative of an increase in the stiffness of the ACE inhibitor treated FPCLs. Indeed, analysis of the FPCL results alone suggested that the enalapril and captopril treated matrices were in fact becoming stiffer with time in culture, with significantly higher peak stresses (compared with day 3 FPCLs) at days 6, 9 and 12, and similar results for equilibrium stresses and H_A (although statistical significance for these parameters

was only achieved on day 9). These large variations in mechanical properties are clearly indicative of a positive effect on matrix stiffness during culture attributable to the presence of the ACE inhibitors.

Although floating FPCLs have been shown to stiffen considerably in culture (Huang *et al*, 1993; Wakatsuki *et al*, 2000), such an effect was not expected in anchored FPCLs because the resistance to deformation as cells infiltrate the matrix means that they are not able to contract the matrix, and therefore cannot increase the collagen density (and thus matrix stiffness) by aggregating fibrils more closely together. In fact, it has been shown that in anchored FPCLs increasing the number of cells results in a weaker matrix with time in culture (Lafrance *et al*, 1995), and it was hypothesised that in the present study the control FPCLs would have become weakened considerably had the experiment been carried out for a longer time, or a greater number of cells been seeded initially.

When values for the equilibrium stress and H_A for the control FPCLs were compared with those from the equivalent cell-free hydrogels, it became apparent that the presence of cells did in fact result in a reduction in the stiffness of the hydrogels. The values at each day for the equilibrium stress and H_A of the control FPCLs were markedly lower, albeit not significantly, than the equivalent values for the cell-free hydrogels. Furthermore, from day 3, the equilibrium stresses and H_A of the cell-free hydrogels showed little sign of variation between the control and ACE inhibitor treated samples, and were more similar to the values from the ACE inhibitor treated FPCLs. There was an apparent increase in equilibrium stresses and H_A for all cell-free hydrogels between day 0 and day 3, but this is probably due to further polymerisation of the collagen during this time period.

In light of these findings it seems reasonable to conclude that enalapril and captopril, rather than imparting additional strength to the gels in the first instance, were in fact preventing the scaffolds from being weakened by the ingrowth of cells. This effect is indicative of the inhibition of collagenolysis, though it does not confirm the mechanism by which this inhibition takes place. Despite evidence suggesting that peak stress values decrease for cell-free hydrogels with increasing time in culture, there were significantly larger values for peak stress towards the end of the culture

period for some of the ACE inhibitor treated cell-free hydrogels when compared with their relative controls, indicating that enalapril and captopril may also impart additional stability to collagen-based scaffolds even in the absence of any collagenolytic enzymes. These findings add weight to the premise explored in **section 3.4.3** that enalapril and captopril may be binding to the collagen, and in doing so stabilising the structure.

It is also of interest that the variation in peak stress, equilibrium stress and H_A between the control and ACE inhibitor treated FPCLs was slightly less pronounced on day 12 than on day 9, suggesting that the stabilising effect of the ACE inhibitors may become less influential with increasing time in culture. This relative reduction in the mechanical properties of the scaffolds towards the end of the experiment could be due to the metabolism of the ACE inhibitors by the cells within the matrix, leading to reduced concentrations of the drugs within the tissue, and consequently a reduced inhibitory effect on collagen degradation. Additionally, if enalapril and captopril truly are intercalated with the collagen in the substrate, then the reduction in mechanical properties on day 12 could also indicate cell activity mediated release of the ACE inhibitors, leading to a reduction in their ability to stabilise the collagen structure. However, it will be necessary to perform further experiments to find out if the ACE inhibitors do indeed bind to the collagen in hydrogels before more definite conclusions can be made.

6.4.4 Conclusions from Mechanical Characterisation of Collagen Hydrogels: Part II

The results presented in this chapter clearly demonstrate that confined compression in combination with biphasic theory is an appropriate and effective technique for assessing the mechanical properties of collagen hydrogels. Although the results in **chapter 5** were not entirely conclusive, modifications to the technique and test equipment, leading to reduced friction and increased sensitivity within the system, vastly improved the ability of the technique to detect variations in the time-dependent properties of the hydrogels.

The technique was clearly able to discriminate between hydrogels of varying collagen concentration, with both test-dependent stress values and material properties (resulting from the application of biphasic theory) exhibiting sensitivity to collagen content. While the stiffness of soft tissues has often been estimated by several different means (**section 1.4.2.1**), methods to estimate their hydraulic permeabilities are far less common, despite the importance of tissue permeability in defining both the mechanical tissue characteristics (Périé *et al*, 2005) and the potential for metabolite transport via convection and diffusion (Ferguson *et al*, 2004; Roberts *et al*, 1996). It is therefore of particular significance that this technique allows for the estimation of k_0, in addition to H_A. Furthermore, although the technique was applied specifically to collagen hydrogels in this instance, it is hypothesised that the technique may also be suitable for the characterisation of other inherently weak hydrated tissues. However, it should also be noted that, for collagen hydrogels, the method used for mechanical characterisation can greatly influence the results (see *Table 6.1*). Such an effect may also be apparent for other hydrated tissues, and care should always be taken to match a testing methodology to a required function as there can be large variations between the stiffness of a tissue in tension or in compression.

Applying the technique and theory to enalapril or captopril treated FPCLs clearly demonstrated the ability for the ACE inhibitors to affect the mechanical properties of the scaffolds. It was initially hypothesised that the stiffness of the anchored FPCLs would decrease with time in culture due to mechanical forces being exerted by the cells as they infiltrate the anchored substrate, weakening it by increasing the tension on collagen fibrils, in addition to substrate weakening by the collagenolytic actions of extruded enzymes. Indeed, such an effect was observed in the present study, with control FPCLs appearing markedly less stiff than the equivalent cell-free hydrogels on each day that measurements were taken. In contrast, enalapril and captopril treated FPCLs retained similar levels of stiffness to the cell-free hydrogels throughout the experiment, demonstrating that the incorporation of ACE inhibitors reduced the ability of the cells to weaken the substrate.

k_0 did not appear affected by the presence of ACE inhibitors, although it was noted that the proliferation of cells upon the scaffolds resulted in a reduction in k_0 following time in culture, with FPCLs demonstrating significantly lower k_0 than the cell-free scaffolds towards the latter part of the study. It was hypothesised that these variations in k_0 of FPCLs were probably due to the cell layer imparting changes to the permeability at the surface boundary of the hydrogels, as opposed to throughout the entire substrate. Consequently, the values obtained in the present study for k_0 of FPCLs were not easily distinguishable from effects imparted by the proliferating cell layer and were not considered a very good approximation of the hydraulic permeability of the whole substrate. If the experiment were to be repeated, it would be useful to seed the cells *within* the substrate, as opposed to on top of it, since a more colloidal distribution of cells would probably result in less severe surface effects.

Despite uncertainties concerning the contribution of the cell layer to permeability calculations, the fact that test-dependent stress values and H_A were significantly higher for enalapril and captopril treated FPCLs than for control FPCLs following time in culture is evidence that the ACE inhibitors were acting to slow down the rate of cell-mediated matrix degradation. Given that enalapril and captopril have already been shown to inhibit enzymatic collagen degradation (**chapter 3**) at non-toxic concentrations (**chapter 4**), it appears likely that these variations in matrix stiffness are attributable to a reduction in the rate of matrix degradation through inhibition of collagenolytic enzyme activities.

The mechanism by which enalapril and captopril inhibit collagen degradation is not yet fully understood, though some of the results from these experiments do further emphasise the hypothesis that the ACE inhibitors may be binding to the collagen, and in doing so stabilising the structure (see **section 3.4.3**). There was some evidence that enalapril and captopril could reduce degradation of the scaffolds even where cells or degradative enzymes were not present. However, further studies are now required to ascertain whether the ACE inhibitors truly are binding to the collagen, and if so, whether they then leach out of the collagen over time, particularly in response to cell ingrowth. Although it has previously been suggested that it is the

presence and activity of cells rather than the chemical composition of a collagen-based matrix that determines its mechanical properties (Saddiq *et al*, 2009), the research presented in this study suggests that incorporating chemicals (specifically inhibitors of collagen degradation) into a matrix can in fact contribute to preserving its mechanical stability over time in culture. Several methods have previously been shown to increase the mechanical strength of collagen-based matrices for tissue engineering, for example through plastic compression (Brown *et al*, 2005) or chemical cross-linking (Osborne *et al*, 1998) of collagen hydrogels. It now remains to be seen whether increasing the strength of matrices using already established techniques, in addition to treatment with ACE inhibitors, could lead to a synergistic effect on the mechanical properties, resulting in scaffolds that are not only initially strong but also have slower, more controllable rates of degradation following cell ingrowth.

It is also possible that the addition of ACE inhibitors to hydrogel scaffolds may be of interest to the field of angiogenesis. Transfer of nutrients into implanted scaffolds is generally diffusion limited, meaning that nutrients are often unable to infiltrate the deeper regions of the implant (Royce *et al*, 1995). Consequently, vascularisation of implants is of utmost importance, as an increase in capillary formation would allow for increased levels of nutrient transfer throughout a scaffold. ACE inhibitors were initially designed to increase blood flow in hypertension via vasodilation of blood vessels (Cushman & Ondetti, 1999), and it is therefore thought that the incorporation of ACE inhibitors into scaffolds for tissue engineering may also have the effect of promoting matrix vascularisation. While this concept can only be considered speculation at this stage, it would be of great interest to investigate the potential of ACE inhibitors to promote matrix vascularisation of tissue scaffolds, having already demonstrated their efficacy in improving the mechanical properties of FPCLs.

Chapter 7

Summary and Further Work

7 Summary and Further Work

7.1 Summary of Thesis Findings

7.1.1 Collagen Degradation Studies

As stated in **section 1.7**, a major aim of this body of research was "to determine whether the ACE inhibitors captopril and enalapril are able to inhibit collagen degradation by collagenase at concentrations that are non-toxic to cells in surrounding tissues". It was therefore of utmost importance to first ascertain whether the selected ACE inhibitors could indeed inhibit collagenase activity at all, and a range of assays were employed to try and quantify any collagenase inhibition that may be taking place.

SDS-PAGE of (rat tail) collagen samples digested with mammalian collagenase from 3T3 fibroblast cell homogenate suggested that both captopril and enalapril were able to inhibit collagen degradation in a dose-dependent fashion, although results using this particular source of collagenase were extremely inconsistent and difficult to replicate, particularly with respect to the ability of the enzymes to degrade the collagen. Consequently, a decision was made to degrade the collagen substrates using bacterial collagenase from *clostridium histolyticum* for all subsequent experiments. This particular type of collagenase, which has been implicated in the progression of tissue necrosis (Harrington, 1996), has a very broad substrate specificity and high activity (compared with mammalian collagenase) (Mookhtiar & Van Wart, 1992), and it was hypothesised that its inhibition by ACE inhibitors could be indicative of a propensity for inhibiting a wide range of proteolytic MMPs, and that the inhibitory effect may be even greater on mammalian collagenases. Experiments using bacterial collagenase provided much more conclusive results.

Collagen substrate zymography was used to analyse the ability of enalapril and captopril to inhibit degradation of the substrate by the proteolytic enzymes within bacterial collagenase. Zymography studies demonstrated that both drugs could inhibit all of the detected proteolytic fractions of bacterial collagenase in a dose

dependent manner, and suggested enalapril to be the more potent inhibitor. However, uncertainties concerning the purity of the collagen within the substrate meant that it was not clear from these results whether the drugs were truly inhibiting collagenase, or were simply inhibiting the degradative action of less specific proteases.

SDS-PAGE analysis clearly demonstrated that both captopril and enalapril were able to inhibit collagen degradation by bacterial collagenase in a dose-dependent manner, with enalapril again appearing the more potent inhibitor. Although statistically significant levels of collagenase inhibition were only achieved at 5 mM concentrations of either drug, the observed irregularities in the results leading to this lack of significance could most likely be attributed to variations between different batches of collagen or collagenase, and there was still clear evidence of collagenase inhibition at concentrations of both captopril and enalapril as low as 0.25 mM.

The results from these studies clearly show that enalapril and captopril are able to inhibit the proteolytic degradation of collagen-based substrates, though they also raised further questions about the potential mechanism or mechanisms of protease inhibition. Some of the results suggested that the ACE inhibitors may actually be binding to the collagen and stabilising it, preserving the native collagen's tightly bound structure, blocking access for degradative enzymes to unwind the triple-helix, or both. These new developments could affect our overall understanding of the mechanisms by which some MMP inhibitors function, since they are commonly thought to bind to the MMPs themselves (Sorbi *et al*, 1993; Yamamoto *et al*, 2007a; Yamamoto *et al*, 2007b; Yamamoto *et al*, 2008).

7.1.2 Cytotoxicity Studies

Having confirmed that both captopril and enalapril could inhibit collagenase, it was then important to determine whether they were able to do so at concentrations that are non-toxic to cells in culture. ACE inhibitors have been used successfully for many years to treat hypertension (Brown & Vaughan, 1998; Ferreira, 2000), and as a consequence there is much literature concerning potential adverse effects and on

their pharmacokinetic and metabolic profiles following therapeutic administration. However, there are few data on the localised effects of high concentrations of ACE inhibitors on cells in culture. Further toxicological testing was therefore considered necessary in this instance, particularly as one of the major aims of the present study was to incorporate the ACE inhibitors into tissue constructs that are designed to interact with cells. Cell viability assays demonstrated that at concentrations of up to 1 mM neither captopril or enalapril are particularly toxic to cells in culture, and that in some cases the presence of either drug may actually increase the metabolic turnover of cells in culture, leading to increased proliferation. Epifluorescent microscopy of fluorescently labelled cells demonstrated that at high (1 mM) concentrations of captopril or enalapril, cells in monolayers and in collagen hydrogels are unaffected in terms of viability, morphology, and actin distribution. It was also noted that at these same ACE inhibitor concentrations fibroblasts were unaffected in their ability to reshape and remodel collagen hydrogel substrates. Consequently it could be assumed that any differences in the mechanical characteristics of FPCLs with and without ACE inhibitors would probably be attributable to the action of collagenolytic enzymes, and not to differences in the mechanical actions of cells within the matrix.

7.1.3 Mechanical Testing

7.1.3.1 Design and Validation of Technique and Theory for Mechanical Characterisation of Collagen Hydrogels

Another of the major aims of the present study was "to develop an effective novel technique for the mechanical characterisation of the time-dependent properties of collagen hydrogel scaffolds". It was intended to try and control the stiffness of the collagen scaffolds through inhibition of collagenase, and therefore it was necessary to find a suitable method to differentiate between scaffolds of varying mechanical properties. Although several methods have been proposed for the mechanical characterisation of hydrogels, the majority of these were considered unsuitable for characterising such inherently weak tissues, and only allow for the extrapolation of a limited set of mechanical parameters (**section 1.4.2.1)**. A decision was made to test

the collagen hydrogels in confined compression, which is non-destructive at small strains, allows for the tests to be performed in standard tissue culture plates, and allows for the application of biphasic theory (Mow *et al*, 1980) to extract physically meaningful parameters. While alternative techniques such as extensometry have been used to measure viscoelasticity in tissues (e.g. Wagenseil *et al* (2003)), the extracted parameters are often not easily related to physical mechanisms. In contrast, biphasic theory (Mow *et al*, 1980) allows for estimation of the stiffness and hydraulic permeability of a substrate. Substrate stiffness has been shown to directly influence cell behaviour (Discher *et al*, 2005; Engler *et al*, 2006; Helary *et al*, 2010), while the hydraulic permeability is known to significantly influence the convective and diffusional properties of solutes through tissue (Pal *et al*, 2009; Riches, 2011) in addition to contributing to the mechanical properties of the tissues. The calculation of these meaningful parameters allows comparisons to be made with a wide range of tissue characterisation methods, whereas test-dependent stress variables (which were also measured) can only be used to compare samples tested using the same particular technique and test setup.

Early efforts to create a system for the repeated testing of sterile samples were abandoned due to insensitivities and friction within the system which led to inaccuracies in the results. Although it was considered beyond the scope of this particular study, it is possible that with appropriate modifications the confined compression technique presented in this study could be used to repeatedly measure variations in the mechanical properties of sterile tissue constructs over time. However, for the purposes of the present study a much simplified version was adopted to ensure that the measurements made were sensitive and accurate.

In order to validate the technique and theory, compression tests were performed on collagen hydrogels of three different collagen concentrations. Both peak and equilibrium stresses demonstrated significant increases relative to collagen content, suggesting that these parameters could be used successfully to characterise mechanical differences in hydrogel properties. The ramp and hold phases during compression were highly typical of poroelastic materials, and consequently biphasic theory was fitted to the whole response. It was hypothesised that as collagen content

increases, the larger fibril density would result in an increase in matrix stiffness and also a reduction in the hydraulic permeability (due to an increase in the viscous drag to fluid flow). These hypotheses were confirmed through experimentation and subsequent analysis, where results demonstrated significant increases in the stiffness and significant decreases in the hydraulic permeabilities of collagen hydrogels as collagen content increased, even where the variations in collagen content were extremely small (0.2%). The results presented in this study suggest that confined compression, together with biphasic theory, is a suitable technique for assessing the mechanical properties of collagen-based hydrogel scaffolds. Material properties and test-dependent experimental stress values exhibited sensitivity to collagen content. Therefore the technique is clearly able to discriminate between hydrogels with small differences in collagen concentration, and potentially may also be suitable for the characterisation of other inherently weak hydrated tissues.

7.1.3.2 Confined Compression Testing of FPCLs

Having confirmed that confined compression, together with biphasic theory (Mow *et al*, 1980), could be used to discriminate between collagen hydrogels with small differences in collagen content, it was hypothesised that the technique may also be sensitive to factors that affect matrix remodelling. Accordingly, the technique was used to compare the effects of captopril and enalapril on the mechanical properties of FPCLs during time in culture. Experimental results clearly demonstrated that, following time in culture, test-dependent stress values and substrate stiffness were significantly higher for captopril and enalapril treated samples than for control samples, while there was no appreciable difference in their hydraulic permeabilities. Despite some problems with cell layers contributing to uncertainties in the calculation of certain parameters for the FPCLs, the results suggest that the ACE inhibitors act to slow down the rate at which cell-seeded matrices are degraded following time in culture. Since captopril and enalapril were already shown to inhibit proteolytic collagen degradation at non-toxic concentrations, and their presence in cell-seeded scaffolds does not affect the ability of cells to remodel the substrate, it seems likely that the perceived increases in matrix stiffness are attributable to a

reduction in the amount of overall substrate degradation following cell ingrowth. It is thought that this effect can be at least partly attributed to the inhibition of collagenolytic enzymes, though further studies are required before the mechanism by which enalapril and captopril inhibit collagen degradation can be fully deduced.

7.1.4 Overall Conclusions from Experimental Work

It has previously been suggested that it is the presence and activity of cells rather than the chemical composition of a collagen-based matrix that determines its mechanical properties (Saddiq *et al*, 2009). However, the research presented in this thesis suggests that the incorporation of certain chemicals, and specifically the incorporation of inhibitors of collagenase, can in fact contribute to the preservation of mechanical stability for such matrices following time in culture. The fact that ACE inhibitors are able to exert this effect is of particular importance as they are widely available drugs that have been used successfully for many years to treat hypertension (Brown & Vaughan, 1998), meaning that they have been approved for medical use by the FDA and are considered safe for therapeutic administration. Furthermore, certain ACE inhibitors have also been shown to stimulate angiogenesis (Fabre *et al*, 1999), and it is thought that, in addition to reducing substrate degradation, the incorporation of ACE inhibitors into tissue engineering scaffolds could also promote scaffold vascularisation, which is necessary for the transfer of nutrients into the deeper regions of scaffolds (Royce *et al*, 1995).

Enalapril and captopril are clearly able to inhibit the degradation of collagen by proteolytic enzymes. However, the mechanism of enzyme inhibition is still not fully understood, and further studies are now required to investigate the hypothesis that the ACE inhibitors may in fact be binding to the collagen, and in doing so stabilising the structure, preventing access for MMPs, or both.

7.1.5 Major Findings of Thesis

The major findings of this thesis are:

- Collagen zymography can be a suitable and effective technique for analysing the activity and inhibition of bacterial collagenase.
- Confined compression in combination with biphasic theory (Mow *et al*, 1980) is a suitable and effective technique for characterising the mechanical properties of collagen hydrogels. The technique is sensitive to small variations in collagen content, and to differences in mechanical properties caused by matrix remodelling.

- The ACE inhibitors captopril and enalapril are able to inhibit collagenase-mediated collagen degradation in a dose-dependent manner. Enalapril appears to be the more potent collagenase inhibitor of the two.
- At concentrations necessary for effective collagenase inhibition, captopril and enalapril are non-toxic to cells in surrounding tissues and do not affect their viability, morphology or activity.
- Treatment with captopril or enalapril slows down the rate at which collagen hydrogels are degraded by mammalian cells, leading to a reduction in the loss of matrix stiffness following time in culture.

7.2 Limitations and Further Work

7.2.1 Collagen Degradation Studies

Bacterial collagenase from *clostridium histolyticum* was used for the majority of the experimental work presented in this thesis, and while it can be assumed that collagen that is cleaved by mammalian collagenase will also be digested by bacterial collagenase (due to the broader substrate specificity and increased levels of activity inherent to the bacterial collagenase (Mookhtiar & Van Wart, 1992)), it would also be of interest to gain further information on the specific ability of the ACE inhibitors to inhibit mammalian collagenase. Although isolation of specific collagenolytic proteins may not be necessary, it is important to note that collagenase is initially secreted by cells in its latent proMMP form (Visse & Nagase, 2003). Accordingly, any further studies using mammalian cells as a source of collagenase for collagen substrate degradation should also include a stage for the activation of the proMMPs, which can be achieved by treatment with APMA (Murphy *et al*, 1977).

7.2.1.1 Collagen Zymography

Collagen zymography was used successfully in the present study to demonstrate bacterial collagenase inhibition by captopril and enalapril. However, a major limitation with the technique was the apparent lack of literature concerning collagen zymography, particularly with respect to its successful application. This lack of resources meant that a concerted effort had to be made to modify and validate the technique before it could be used with confidence to observe and quantify collagenase inhibition.

The technique was clearly able to detect bacterial collagenase activity, though there were some issues surrounding the purity of the collagen substrate, and it was considered possible that the zymograms may have contained collagen that was already partially denatured and therefore susceptible to degradation by other MMPs that are less specific than true collagenases. The bacterial collagenase used contains a mixture of ECM-degrading enzymes, and it was not entirely clear from the

zymography results whether enalapril and captopril are truly inhibitors of collagenase or were simply inhibiting the degradative action of less specific proteases. It was originally hypothesised that running the zymograms at 4 °C provided less usable results, but it has since been realised that performing the experiments at this low temperature is more likely to ensure that the collagen within the polyacrylamide gels remains in its native form. Accordingly, it would be of interest to perform more zymography experiments with ACE inhibitors, but where the zymograms are run at a lower temperature in order to further ensure the specificity of the substrate for true collagen cleaving enzymes. Nevertheless, the zymography results presented in this thesis provide conclusive evidence that both captopril and enalapril can inhibit substrate degradation by bacterial collagenase.

Given more time it would also have been useful to test a wider range of potential collagenase inhibitor concentrations, especially in the case of EDTA (used as a positive control) where even the lowest tested concentration provided total inhibition of collagen degradation. It would also have been useful to check whether the incorporation of ACE inhibitors directly into the zymography gel (as opposed to being dissolved in the incubation buffer (see **section 3.2.2.3**)) would still inhibit proteolysis; particularly as the ACE inhibitors were ultimately incorporated into hydrogel scaffolds. Furthermore, as mentioned previously, it would also be of interest to perform collagen zymography experiments using APMA-activated collagenase from mammalian sources, in addition to the bacterial collagenase used in the present study.

7.2.1.2 SDS-PAGE

In the present study it was demonstrated using SDS-PAGE that the ACE inhibitors captopril and enalapril are able to inhibit the degradation of collagen by collagenolytic enzymes. However, there were also problems with inconsistencies and irregularities in some of the results from the collagen degradation studies, and it is supposed that these probably resulted from variation between different batches of collagen or collagenase. It is therefore important to ensure that for all similar studies

special care is taken to guarantee consistency between different batches of collagen or collagenase. If possible the same batches of collagen and collagenase should be used for all associated experiments.

7.2.2 Cytotoxicity Studies

The cytotoxicity studies presented in this thesis demonstrate that both captopril and enalapril are non-toxic to cells in culture at concentrations that inhibit collagenase. Although the results are extensive and conclusive, it should be noted that only one cell-line was used for all experiments (3T3 mouse fibroblasts), and therefore it would be of interest to perform toxicity studies with additional cell-lines. As tissue engineered scaffolds are ultimately designed to be implanted into humans, it would be particularly appropriate to also carry out cell studies using human cells, either from immortalised cell lines or primary cell cultures.

7.2.3 Mechanical Characterisation of Collagen Hydrogels

For the purposes of this thesis a novel method for characterising the time-dependent properties of collagen hydrogels was designed, implemented and validated. This method was based upon the confined compression of collagen hydrogels and the application of biphasic theory (Mow *et al*, 1980) to extract meaningful mechanical parameters. The technique was then used successfully to characterise differences in the mechanical properties of FPCLs treated with either captopril or enalapril. The main limitations encountered during the early stages of testing were a consequence of using dated equipment, and specifically the insensitivities of the 1 kN load cell being utilised. Thankfully, the later availability of a BOSE ElectroForce® 3200 Test Instrument and more sensitive load cells meant that many of the initial limitations of accuracy and sensitivity became much less problematic.

Early efforts to create an enclosed device for confined compression testing of sterile samples were unsuccessful, due primarily to large amounts of friction within the

system leading to erroneous measurements. Although this particular approach was abandoned, it is possible that with appropriate modifications such a system could be successful. However, if the same samples were to be tested at various time points, great care would have to be taken to ensure adequate controls, especially when testing cell-seeded constructs, as mechanical loading of cells in tissue constructs has been shown to influence protease production (Prajapati *et al*, 2000). It is also possible that samples could experience some level of permanent deformation following compressions. A much simplified approach was employed for the majority of mechanical testing, and while this approach provided extremely useful results, it is possible that the accuracy of the system could have been improved by making further refinements to the test setup. One particular modification that would improve the overall sensitivity of the system would be to manufacture a lower weight porous indenter, which would in turn allow for the use of a lower rated (and therefore more sensitive) load cell. However, the 10 N load cell was of sufficient sensitivity for the purposes of this thesis.

There were also issues with fluid trapped beneath the floating hydrogels causing errors in measurements, and ultimately leading to a requirement for excess fluid to be removed immediately prior to testing. Although the removal of this excess fluid did not appear to cause much variation in recorded stress responses, it would be ideal if samples could be tested in fluid as such conditions would be more similar to those present *in vivo*. It would be useful if steps could be taken to ensure that no fluid is trapped beneath the hydrogel, while still allowing for samples to be tested fully submerged.

During the present study the proliferating cell layer on top of the hydrogels appeared to have substantial effects on the calculated values for k_0, which limited the interpretation of certain mechanical properties for FPCLs. Since these variations in k_0 were probably due to the cell layer imparting localised changes to the permeability at the surface boundary of the hydrogels (as opposed to throughout the entire substrate), it is thought that seeding cells within the substrate, rather than on top of it, would result in less severe surface effects and provide more informative results.

Having confirmed that confined compression in combination with biphasic theory (Mow *et al*, 1980) is a suitable and effective technique for characterising the mechanical properties of collagen hydrogels, it would interesting to test the efficacy of the technique for characterising other inherently weak, hydrated tissues. Many different types of hydrogel have been proposed for tissue engineering purposes (Lee & Mooney, 2001), and the confined compression technique presented in this thesis may be applicable to many more hydrogel compositions than simply those that are collagen-based.

With respect to biphasic theory, it is important to be aware that the linear biphasic model (Mow *et al*, 1980) adopted in this study is the simplest form of biphasic model. Further characterisation using more complex finite deformation models, which describe how both permeability and matrix stiffness vary with strain, could elucidate the mechanical behaviour of the collagen hydrogels more clearly. Additionally, there are poroviscoelastic models (for example Suh & DiSilvestro (1999)), in which the solid phase is modelled as a viscoelastic solid, whilst maintaining the solid-fluid interactions of the biphasic model. Collagen can be considered to be a viscoelastic solid at the fibril level (Shen *et al*, 2011), and thus poroviscoelastic model may be well suited for the characterisation of collagen-based hydrogels.

It would also be interesting to perform further investigations into the importance of the peak compressive stress with respect to material properties, as this variable demonstrated the most sensitivity to the experimental conditions during the present study. Since H_A is dependent on the stress equilibrium point as $t \rightarrow \infty$ (which is never achieved), and is then used in the determination of k_0 and M, inverse methods may lead to difficulties in the precise determination of these variables. For a given ramp speed and a specific sample height, it can be noted from Eq. (7) (see **section 6.1.2**) that the peak stress is dependent on $1/k$ and H_A. It would therefore be useful to perform a series of experiments to determine the relationship between the peak compressive stress and the strain rate and sample height, as peak stress may in fact be a simple and easily derivable quantity.

7.2.4 Future Work

The results presented in this thesis demonstrate that captopril and enalapril are able to inhibit collagenase at concentrations that are non-toxic to cells in surrounding tissues, and that the incorporation of these ACE inhibitors into FPCLs results in a stiffer matrix with time in culture. Although the outcomes of this thesis can be considered successful, there is a great deal of further work in the area that can now be undertaken in response to the results presented.

The mechanisms by which enalapril and captopril inhibit collagen degradation by MMPs is still not fully understood, although during the present study there was evidence that the ACE inhibitors may in fact be binding to the collagen, and in doing so stabilising its structure. Therefore it would be worthwhile to perform further experiments to ascertain whether the drugs truly do become intercalated with the collagen in a substrate. It would be particularly useful to find out if there is much retention of the ACE inhibitors in collagen hydrogels following time in culture, and if so, whether the release of the intercalated drugs is affected in any way by cell ingrowth or MMP activities.

Only two ACE inhibitors, captopril and enalapril, were chosen for study in this project, and it would be interesting to find out if a wider range of ACE inhibitors also have the ability to stabilise and stiffen tissue engineered collagen at non-toxic concentrations, and if there are variations in their potency in this respect. As discussed in **section 1.5.4**, a number of synthetic MMP inhibitors have been designed, and it would also be interesting to determine whether such agents have a similar ability to impart mechanical stability to tissue engineered collagen constructs at non-toxic levels.

Tissue engineered constructs are ultimately intended to be implanted into humans, and as such it is extremely important that the *in vivo* compatibility of collagen containing ACE inhibitors is ascertained. Therefore animal studies will be required if the technology is to be taken further. Such studies would be used to confirm whether the incorporation of ACE inhibitors into collagen scaffolds can slow down and control the rate of collagen degradation *in vivo*, in addition to obtaining further

information about the potential *in vivo* toxicology of localised high levels of ACE inhibitors in tissues. As some ACE inhibitors have been said to stimulate angiogenesis (Fabre *et al*, 1999), it would also be useful to check for angiogenic variations in implanted scaffolds, in addition to variations in their degradative profiles.

Several methods have already been shown to increase the mechanical strength of collagen-based matrices for tissue engineering, such as plastic compression (Brown *et al*, 2005) or chemical cross-linking (Osborne *et al*, 1998). It now remains to be seen whether increasing the strength of collagen-based matrices using these already established techniques, in addition to treatment with ACE inhibitors, could result in scaffolds that are not only initially strong, but also have slower, more controllable rates of degradation following implantation.

7.3 Implications for the Technologies Developed in the Present Study

7.3.1 Mechanical Characterisation of Hydrogels

For the purposes of this thesis, a technique was developed for the mechanical characterisation of collagen hydrogel scaffolds. Several techniques for the mechanical characterisation of hydrogel scaffolds have already been proposed (**section 1.4.2.1)**, but it is the author's opinion that the technique presented in this thesis offers several distinct advantages over other commonly used methods. One major advantage is the simplicity with which tests can be performed, assuming that a sensitive and appropriately controlled load cell is available. Where scaffolds are moulded in cylindrical wells (as was the case for the present study), they can be tested without being moved first, meaning that disruption to samples is kept to a minimum. The technique also allows for hydrogels to be characterised in a relatively short time, and so a high throughput of samples is possible. The technique is also non-destructive, and it is likely that with further modifications it could be used to characterise samples without compromising sterility. However, the main advantage of the technique is the ability to characterise the time-dependent properties of the

materials, and specifically to extract meaningful parameters that are not exclusive to the particular test being performed. These stated advantages, together with the potential for confined compression and biphasic theory (Mow *et al*, 1980) to be used to characterise a range of different types of hydrogel scaffolds, mean that the technique presented in this thesis is likely to be of great value to anyone interested in the mechanical characterisation of hydrogels and soft tissues.

7.3.2 MMP Inhibition By ACE Inhibitors

It has been known for some time that some ACE inhibitors are also able to inhibit certain MMPs. However, previous studies have focussed primarily on the inhibition of MMP-2 and MMP-9 by ACE inhibitors (for example Jin *et al* (2007); Sorbi *et al* (1993); Yamamoto *et al* (2008)). In contrast, the present study focussed on the ability of ACE inhibitors to inhibit collagen-cleaving MMPs, for which they proved successful. Therefore it seems likely that ACE inhibitors have a broad specificity for MMP inhibition. Evidence also arose during the present study of another mechanism by which ACE inhibitors may impede collagen degradation, suggesting that they may in fact bind to the collagen (as opposed to the MMPs), stabilising the structure, blocking access for MMPs, or both.

This new information about the inhibition of collagen degradation by ACE inhibitors, in addition to the potential for the drugs to bind to and stabilise structural proteins, has implications for any research concerning MMP inhibition. Although the main focus of MMP inhibitor research to date has been in the fields of cancer therapy and cardiovascular disorders, the research presented in this thesis suggests that there are also many potential therapeutic applications in other areas, such as tissue engineering and wound repair.

7.3.3 ACE Inhibitor Treated Collagen Scaffolds

The potential to slow down the rate at which collagen-based scaffolds are degraded by the cells of the body has many implications. For tissue engineering applications, slowing down the rate at which an implanted scaffold degrades would allow more time for the relevant cells to create a new matrix of native tissue. A slower rate of collagen degradation would also be extremely desirable where the scaffold is intended to be used as a space-filling agent, which in turn would be of particular interest to the cosmetics industry, where collagen implants have been successfully used for many years to enhance personal appearance and challenge the aging process (Baumann *et al*, 2006). By slowing down the rate at which implants degrade they would not have to be re-implanted as regularly to maintain repair or cosmetic effect. The fact that enalapril and captopril are able to inhibit bacterial collagenase activity is also highly relevant to the field of tissue engineering, since bacterial collagenases are implicated in the progression of many human necrotic diseases (Harrington, 1996). The most successful applications of tissue engineered collagen to date have been for the treatment of necrotic conditions such as venous leg and diabetic foot ulcers (Allie *et al*, 2004; Falanga, 1998; Kirsner, 1998), and it is possible that the inclusion of factors which inhibit non-specific collagenase activity could ultimately lead to longer-lasting and more stable constructs for the repair of necrotising tissues. The technology could also benefit applications for the controlled release of drugs and other soluble factors, as slowing down the rate of substrate degradation may allow even more control over the rate at which these soluble factors are released into surrounding tissues.

It is important to note that the ACE inhibitor treatment presented in this thesis is applied before the gelation stage, and thus for hydrogel applications collagen treated in this manner would still be mouldable or injectable. The fact that treatment with ACE inhibitors does not affect the ability of collagen solutions to form a hydrogel means that the technology may be of benefit to any application requiring the use of such hydrogels. Therefore, the potential to increase the strength and stability of collagen implants by treating them with readily available and FDA approved ACE

inhibitors has major implications throughout the fields of tissue engineering, regenerative medicine and cosmetic surgery.

7.4 Final Words

The research presented in this thesis, and the resulting contribution to knowledge and understanding, will be of benefit to many different aspects of bioengineering, which in itself is profoundly multidisciplinary. This thesis has contributed knowledge to several of these different disciplines, including cell studies, toxicology, the study of biomaterials and biomechanics. While each of these disciplines may be studied in their own right, it is important to remember that they are often interconnected, and that biological processes should not be expected to occur autonomously. In order to fully understand how a particular process works, it is often a good idea to take a few steps back and look at the bigger picture.

PUBLICATIONS

Papers published in peer reviewed journals:

Busby GA, Grant MH, MacKay SP, Riches PE (2012). Confined Compression of Collagen Hydrogels. *Journal of Biomechanics* in press.

Abstracts published in peer reviewed journals – presented at conferences:

Busby GA, Riches P, MacKay S, Grant MH (2011) Culture of 3T3 fibroblasts in collagen gels in the presence of ACE inhibitors. *Toxicology* **290**(2)**:** 135-136

Busby GA, Riches P, MacKay S, Grant MH (2011) A novel method for characterising mechanical properties of hydrogels. *European Cells and Materials* **22**(3)**:** 70

Conference proceedings:

Busby GA, Riches P, MacKay S, Grant MH (2011) Compression testing of collagen hydrogels. Proceedings of BOSE ElectroForce Systems Group UK User Group Meeting (London).

Busby GA, Riches P, MacKay S, Grant MH (2011) Fibroblast-populated collagen lattices in the presence of ACE inhibitors. Proceedings of Scottish Toxicology Interest Group Meeting (Glasgow).

Unpublished conference abstracts:

Busby GA, Riches P, MacKay S, Grant MH (2011) A Novel Method of Characterising Mechanical Properties of Collagen Based Scaffolds for Tissue Engineering. Presented at UK Biomaterials Society (Glasgow).

REFERENCES

Abraham GA, Murray J, Billiar K, Sullivan SJ (2000) Evaluation of the porcine intestinal collagen layer as a biomaterial. *Journal of Biomedical Materials Research* **51**(3)**:** 442-452

Ahearne M, Yang Y, El Haj AJ, Then KY, Liu K-K (2005) Characterizing the viscoelastic properties of thin hydrogel-based constructs for tissue engineering applications. *Journal of The Royal Society Interface* **2**(5)**:** 455-463

Ahearne M, Yang Y, Then KY, Liu KK (2008) Non-destructive mechanical characterisation of UVA/riboflavin crosslinked collagen hydrogels. *Br J Ophthalmol* **92**(2)**:** 268-271

Al-Furaih TA, McElnay JC, Elborn JS, Rusk R, Scott MG, McMahon J, Nicholls DP (1991) Sublingual captopril — a pharmacokinetic and pharmacodynamic evaluation. *European Journal of Clinical Pharmacology* (4)**:** 393-398

Allen JW, Hassanein T, Bhatia SN (2001) Advances in bioartificial liver devices. *Hepatology* **34**(3)**:** 447-455

Allie DE, Hebert CJ, Lirtzman MD, Wyatt CH, Keller VA, Souther SM, Allie AA, Mitran EV, Walker CM (2004) Novel treatment strategy for leg and sternal wound complications after coronary artery bypass graft surgery: Bioengineered Apligraf. *The Annals of Thoracic Surgery* **78**(2)**:** 673-678

Amaral KF, Rogero MM, Fock RA, Borelli P, Gavini G (2007) Cytotoxicity analysis of EDTA and citric acid applied on murine resident macrophages culture. *International Endodontic Journal* **40**(5)**:** 338-343

Anseth KS, Bowman CN, Brannon-Peppas L (1996) Mechanical properties of hydrogels and their experimental determination. *Biomaterials* **17**(17)**:** 1647-1657

Ateshian GA, Warden WH, Kim JJ, Grelsamer RP, Mow VC (1997) Finite deformation biphasic material properties of bovine articular cartilage from confined compression experiments. *Journal of Biomechanics* **30**(11-12)**:** 1157-1164

Baba T, Murabayashi S, Tomiyama T, Takebe K (1990) The pharmacokinetics of enalapril in patients with compensated liver cirrhosis. *British Journal of Clinical Pharmacology* **29**(6)**:** 766-9

Babensee JE, Anderson JM, McIntire LV, Mikos AG (1998) Host response to tissue engineered devices. *Advanced Drug Delivery Reviews* **33**(1–2)**:** 111-139

Badylak SF (2004) Xenogeneic extracellular matrix as a scaffold for tissue reconstruction. *Transplant Immunology* **12**(3–4)**:** 367-377

Badylak SF (2007) The extracellular matrix as a biologic scaffold material. *Biomaterials* **28**(25)**:** 3587-3593

Ballal NV, Kundabala M, Bhat S, Rao N, Rao BSS (2009) A comparative in vitro evaluation of cytotoxic effects of EDTA and maleic acid: Root canal irrigants. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology* **108**(4)**:** 633-638

Barocas VH, Moon AG, Tranquillo RT (1995) The fibroblast-populated collagen microsphere assay of cell traction force---part 2: measurement of the cell traction parameter. *Journal of Biomechanical Engineering* **117**(2)**:** 161-170

Barocas VH, Tranquillo RT (1997) An anisotropic biphasic theory of tissue-equivalent mechanics: the interplay among cell traction, fibrillar network deformation, fibril alignment, and cell contact guidance. *Journal of Biomechanical Engineering* **119**(2)**:** 137-145

Baumann L, Kaufman J, Saghari S (2006) Collagen fillers. *Dermatologic Therapy* **19**(3)**:** 134-140

Behravesh E, Timmer MD, Lemoine JJ, Liebschner MAK, Mikos AG (2002) Evaluation of the in vitro degradation of macroporous hydrogels using gravimetry, confined compression testing, and microcomputed tomography. *Biomacromolecules* **3**(6)**:** 1263-1270

Bell E, Ivarsson B, Merrill C (1979) Production of a tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential in vitro. *Proceedings of the National Academy of Sciences* **76**(3)**:** 1274-1278

Biollaz J, Schelling JL, Jacot Des Combes B, Brunner DB, Desponds G, Brunner HR, Ulm EH, Hichens M, Gomez HJ (1982) Enalapril maleate and a lysine analogue (MK-521) in normal volunteers; relationship between plasma drug levels and the renin angiotensin system. *British Journal of Clinical Pharmacology* **14**(3)**:** 363-8

Biot MA (1956) General solutions of the equations of elasticity and consolidation for a porous material. *Journal of Applied Mechanics* **23**: 91-96

Biot MA (1973) Nonlinear and semilinear rheology of porous solids. *Journal of Geophysical Research* **78**(23)**:** 4924-4937

Bode W, Fernandez-Catalan C, Grams F, Gomis-Rüth FX, Nagase H, Tschesche H, Maskos K (1999a) Insights into MMP-TIMP interactions. *Annals of the New York Academy of Sciences* **878**(1)**:** 73-91

Bode W, Fernandez-Catalan C, Tschesche H, Grams F, Nagase H, Maskos K (1999b) Structural properties of matrix metalloproteinases. *Cellular and Molecular Life Sciences* **55**(4)**:** 639-652

Borenfreund E, Puerner JA (1985) Toxicity determined in vitro by morphological alterations and neutral red absorption. *Toxicology Letters* **24**(2-3)**:** 119-24

Brandl F, Sommer F, Goepferich A (2007) Rational design of hydrogels for tissue engineering: Impact of physical factors on cell behavior. *Biomaterials* **28**(2)**:** 134-146

Briscoe BJ, Panesar SS (1991) The application of the blister test to an elastomeric adhesive. *Proceedings of the Royal Society of London Series A: Mathematical and Physical Sciences* **433**(1887)**:** 23-43

Brower GL, Levick SP, Janicki JS (2007) Inhibition of matrix metalloproteinase activity by ACE inhibitors prevents left ventricular remodeling in a rat model of heart failure. *American Journal of Physiology - Heart and Circulatory Physiology* **292**(6)**:** 3057-3064

Brown E, Dejana E (2003) Cell-to-cell contact and extracellular matrix. *Current Opinions in Cell Biology* **15:** 505-508

Brown NJ, Vaughan DE (1998) Angiotensin-converting enzyme inhibitors. *Circulation* **97**(14)**:** 1411-1420

Brown PD, Bloxidge RE, Stuart NSA, Galler KC, Carmichael J (1993) Association between expression of activated 72-kilodalton gelatinase and tumor spread in non-small-cell lung carcinoma. *Journal of the National Cancer Institute* **85**(7)**:** 574-578

Brown RA, Phillips JB (2007) Cell responses to biomimetic protein scaffolds used in tissue repair and engineering. *International Review of Cytology*, **262:** 75-150

Brown RA, Wiseman M, Chuo CB, Cheema U, Nazhat SN (2005) Ultrarapid engineering of biomimetic materials and tissues: fabrication of nano- and microstructures by plastic compression. *Advanced Functional Materials* **15**(11)**:** 1762-1770

Cawston TE, Rowan A (1998) Prevention of cartilage breakdown by matrix metalloproteinase inhibition--a realistic therapeutic target? *Rheumatology* **37**(4)**:** 353-356

Celada F, Rotman B (1967) A fluorochromatic test for immunocytotoxicity against tumor cells and leucocytes in agarose plates. *Proceedings of the National Academy of Sciences of the United States of America* **57**(3)**:** 630-6

Chen G, Ushida T, Tateishi T (2002) Scaffold design for tissue engineering. *Macromolecular Bioscience* **2**(2)**:** 67-77

Chen M, Zamora PO, Som P, Peña LA, Osaki S (2003) Cell attachment and biocompatibility of polytetrafluoroethylene (PTFE) treated with glow-discharge

plasma of mixed ammonia and oxygen. *Journal of Biomaterials Science, Polymer Edition* **14**(9)**:** 917-935

Chiou S-H, Wu S-H (1999) Evaluation of commonly used electrophoretic methods for the analysis of proteins and peptides and their application to biotechnology. *Analytica Chimica Acta* **383**(1-2)**:** 47-60

Chen CZ, Raghunath M (2009) Focus on collagen: *in vitro* systems to study fibrogenesis and antifibrosis – state of the art. *Fibrogenesis Tissue Repair* **2:**(7)

Chung L, Dinakarpandian D, Yoshida N, Lauer-Fields JL, Fields GB, Visse R, Nagase H (2004) Collagenase unwinds triple-helical collagen prior to peptide bond hydrolysis. *The EMBO Journal* **23**(15)**:** 3020-3030

Clark CK (1995) Implantable biohybrid artificial organs. *Cell Transplantation* **4**(4)**:** 415-436

Coussens LM, Fingleton B, Matrisian LM (2002) Matrix metalloproteinase inhibitors and cancer—trials and tribulations. *Science* **295**(5564)**:** 2387-2392

Coviello T, Coluzzi G, Palleschi A, Grassi M, Santucci E, Alhaique F (2003) Structural and rheological characterization of Scleroglucan/borax hydrogel for drug delivery. *International Journal of Biological Macromolecules* **32**(3–5)**:** 83-92

Cushman DW, Ondetti MA (1999) Design of angiotensin converting enzyme inhibitors. *Nature Medicine* **5**(10)**:** 1110-1112

Darcy HPG (1856) Les fontaines publiques de la Ville de Dijon. Paris, France: Victon Dalmont

Davis BJ (1964) Disc electrophoresis–II Method and application to human serum proteins. *Annals of the New York Academy of Sciences* **121**(2)**:** 404-427

DiBianco R (1986) Adverse reactions with angiotensin converting enzyme (ACE) inhibitors. *Medical toxicology* **1**(2)**:** 122-41

Discher DE, Janmey P, Wang Y-l (2005) Tissue cells feel and respond to the stiffness of their substrate. *Science* **310**(5751)**:** 1139-1143

Drury JL, Mooney DJ (2003) Hydrogels for tissue engineering: Scaffold design variables and applications. *Biomaterials* **24**(24)**:** 4337-4351

Eastwood M, Mudera VC, McGrouther DA, Brown RA (1998) Effect of precise mechanical loading on fibroblast populated collagen lattices: Morphological changes. *Cell Motility and the Cytoskeleton* **40**(1)**:** 13-21

Edidin M (1970) A Rapid, Quantitative fluorescence assay for cell damage by cytotoxic antibodies. *The Journal of Immunology* **104**(5)**:** 1303-1306

Egeblad M, Werb Z (2002) New functions for the matrix metalloproteinases in cancer progression. *Nature Reviews Cancer* **2**(3)**:** 161-174

Elliott S, Cawston T (2001) The clinical potential of matrix metalloproteinase inhibitors in the rheumatic disorders. *Drugs & Aging* **18**(2)**:** 87-99

Elsdale T, Bard J (1972) Collagen substrata for studies on cell behavior. *The Journal of Cell Biology* **54**(3)**:** 626-637

Engler AJ, Sen S, Sweeney HL, Discher DE (2006) Matrix elasticity directs stem cell lineage specification. *Cell* **126**(4)**:** 677-689

Even-Ram S, Yamada KM (2005) Cell migration in 3D matrix. *Current Opinion in Cell Biology* **17**(5)**:** 524-532

Fabre J-E, Rivard A, Magner M, Silver M, Isner JM (1999) Tissue inhibition of angiotensin-converting enzyme activity stimulates angiogenesis in vivo. *Circulation* **99**(23)**:** 3043-3049

Falanga V (1998) Apligraf treatment of venous ulcers and other chronic wounds. *Journal of Dermatology* **25**(12)**:** 812-7

Feng Z, Matsumoto T, Nakamura T (2003a) Measurements of the mechanical properties of contracted collagen gels populated with rat fibroblasts or cardiomyocytes. *Journal of Artificial Organs* **6**(3)**:** 192-196

Feng Z, Yamato M, Akutsu T, Nakamura T, Okano T, Umezu M (2003b) Investigation on the mechanical properties of contracted collagen gels as a scaffold for tissue engineering. *Artificial Organs* **27**(1)**:** 84-91

Ferguson SJ, Ito K, Nolte L-P (2004) Fluid flow and convective transport of solutes within the intervertebral disc. *Journal of Biomechanics* **37**(2)**:** 213-221

Ferreira SH (2000) Angiotensin converting enzyme: History and relevance. *Seminars in Perinatology* **24**(1)**:** 7-10

Foglieni C, Meoni C, Davalli AM (2001) Fluorescent dyes for cell viability: an application on prefixed conditions. *Histochemistry and Cell Biology* **115**(3)**:** 223-229

Frederick Woessner Jr J (1995) Quantification of matrix metalloproteinases in tissue samples. *Methods in Enzymology* **248:** 510-528

Friess W (1998) Collagen – biomaterial for drug delivery. *European Journal of Pharmaceutics and Biopharmaceutics* **45**(2)**:** 113-136

Gelse K, Pöschl E, Aigner T (2003) Collagens—structure, function, and biosynthesis. *Advanced Drug Delivery Reviews* **55**(12)**:** 1531-1546

Gentleman E, Lay AN, Dickerson DA, Nauman EA, Livesay GA, Dee KC (2003) Mechanical characterization of collagen fibers and scaffolds for tissue engineering. *Biomaterials* **24**(21)**:** 3805-3813

Gentleman E, Livesay G, Dee K, Nauman E (2006) Development of ligament-like structural organization and properties in cell-seeded collagen scaffolds. *Annals of Biomedical Engineering* **34**(5)**:** 726-736

Gentleman E, Nauman EA, Dee KC, Livesay GA (2004) Short collagen fibers provide control of contraction and permeability in fibroblast-seeded collagen gels. *Tissue Engineering* **10**(3-4)**:** 421-7

Gilbert TW, Sellaro TL, Badylak SF (2006) Decellularization of tissues and organs. *Biomaterials* **27**(19)**:** 3675-3683

Gilding, D. K. (Williams, D. F., ed.) (1981) *Biocompatibility of Clinical Implant Materials*, CRC Press. 209–232

Gogly B, Groult N, Hornebeck W, Godeau G, Pellat B (1998) Collagen zymography as a sensitive and specific technique for the determination of subpicogram levels of interstitial collagenase. *Analytical Biochemistry* **255**(2)**:** 211-216

Goo HC, Hwang Y-S, Choi YR, Cho HN, Suh H (2003) Development of collagenase-resistant collagen and its interaction with adult human dermal fibroblasts. *Biomaterials* **24**(28)**:** 5099-5113

Grant C, Twigg P, Egan A, Moody A, Smith A, Eagland D, Crowther N, Britland S (2006) Poly(vinyl alcohol) hydrogel as a biocompatible viscoelastic mimetic for articular cartilage. *Biotechnology Progress* **22**(5)**:** 1400-1406

Grillo HC, Gross J (1962) Thermal reconstitution of collagen from solution and the response to its heterologous implantation. *Journal of Surgical Research* **2:** 69-82

Grinnell F (1994) Fibroblasts, myofibroblasts, and wound contraction. *Journal of Cell Biology* **124**(4)**:** 401-4

Grinnell F (2003) Fibroblast biology in three-dimensional collagen matrices. *Trends in Cell Biology* **13**(5)**:** 264-269

Gross J, Kirk D (1958) The heat precipitation of collagen from neutral salt solutions: some rate-regulating factors. *The Journal of biological chemistry* **233**(2)**:** 355-60

Gu WY, Yao H, Huang CY, Cheung HS (2003) New insight into deformation-dependent hydraulic permeability of gels and cartilage, and dynamic behavior of agarose gels in confined compression. *Journal of Biomechanics* **36**(4)**:** 593-598

Gunatillake PA, Adhikari R (2003) Biodegradable synthetic polymers for tissue engineering. *European Cells and Materials* **5:** 1-16

Guo X-G, Uzui H, Mizuguchi T, Ueda T, Chen J-z, Lee J-D (2008) Imidaprilat inhibits matrix metalloproteinase-2 activity in human cardiac fibroblasts induced by interleukin-1β via NO-dependent pathway. *International Journal of Cardiology* **126**(3)**:** 414-420

Harkness RD (1961) Biological functions of collagen. *Biological Reviews* **36**(4)**:** 399-455

Harrington DJ (1996) Bacterial collagenases and collagen-degrading enzymes and their potential role in human disease. *Infection and Immunity* **64**(6): 1885-1891

Helary C, Bataille I, Abed A, Illoul C, Anglo A, Louedec L, Letourneur D, Meddahi-Pellé A, Giraud-Guille MM (2010) Concentrated collagen hydrogels as dermal substitutes. *Biomaterials* **31**(3)**:** 481-490

Heussen C, Dowdle EB (1980) Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. *Analytical Biochemistry* **102**(1)**:** 196-202

Holmes MH (1986) Finite Deformation of soft tissue: Analysis of a mixture model in uni-axial compression. *Journal of Biomechanical Engineering* **108**(4)**:** 372-381

Huang D, Chang T, Aggarwal A, Lee R, Ehrlich H (1993) Mechanisms and dynamics of mechanical strengthening in ligament-equivalent fibroblast-populated collagen matrices. *Annals of Biomedical Engineering* **21**(3)**:** 289-305

Huang S, Ingber DE (1999) The structural and mechanical complexity of cell-growth control. *Nature Cell Biology* **1**(5)**:** E131-E138

Iatridis JC, Setton LA, Foster RJ, Rawlins BA, Weidenbaum M, Mow VC (1998) Degeneration affects the anisotropic and nonlinear behaviors of human anulus fibrosus in compression. *Journal of biomechanics* **31**(6)**:** 535-544

Invitrogen™ (2011) Gibco® Collagenase, Type II, powder (*http://products.invitrogen.com/ivgn/product/17101015*). UK: Life Technologies™

Jiang H, Su W, Mather PT, Bunning TJ (1999) Rheology of highly swollen chitosan/polyacrylate hydrogels. *Polymer* **40**(16)**:** 4593-4602

Jin Y, Han H-C, Lindsey ML (2007) ACE inhibitors to block MMP-9 activity: New functions for old inhibitors. *Journal of molecular and cellular cardiology* **43**(6)**:** 664-666

Johnson WH, Roberts NA, Borkakoti N (1987) Collagenase inhibitors: their design and potential therapeutic use. *J Enzyme Inhib* **2**(1)**:** 1-22

Johnston CI, Jackson BJ, Larmour I, Cubella R, Casley D (1984) Plasma enalapril levels and hormonal effects after short- and long-term administration in essential hypertension. *British Journal of Clinical Pharmacology* **18(2):** 233-239

Kadler KE, Baldock C, Bella J, Boot-Handford RP (2007) Collagens at a glance. *Journal of Cell Science* **120(12):** 1955-8

Kadler KE, Holmes DF, Trotter JA, Chapman JA (1996) Collagen fibril formation. *The Biochemical journal* **316 (1):** 1-11

Kim B-S, Mooney DJ (1998) Development of biocompatible synthetic extracellular matrices for tissue engineering. *Trends in Biotechnology* **16**(5)**:** 224-230

Kirsner RS (1998) The use of Apligraf in acute wounds. *Journal of Dermatology* **25**(12)**:** 805-11

Kleiner DE, Stetlerstevenson WG (1994) Quantitative zymography: Detection of picogram quantities of gelatinases. *Analytical Biochemistry* **218**(2)**:** 325-329

Knapp DM, Barocas VH, Moon AG, Yoo K, Petzold LR, Tranquillo RT (1997) Rheology of reconstituted type I collagen gel in confined compression. *Journal of Rheology* **41**(5)**:** 971-993

Koob TJ, Hernandez DJ (2003) Mechanical and thermal properties of novel polymerized NDGA–gelatin hydrogels. *Biomaterials* **24**(7)**:** 1285-1292

Korhonen RK, Laasanen MS, Töyräs J, Rieppo J, Hirvonen J, Helminen HJ, Jurvelin JS (2002) Comparison of the equilibrium response of articular cartilage in unconfined compression, confined compression and indentation. *Journal of Biomechanics* **35**(7)**:** 903-909

Krishnan L, Weiss JA, Wessman MD, Hoying JB (2004) Design and application of a test system for viscoelastic characterization of collagen gels. *Tissue Engineering* **10**(1-2)**:** 241-52

Kühn K (1985) Structure and biochemistry of collagen. *Aesthetic Plastic Surgery* **9**(2)**:** 141-144

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**(5259)**:** 680-5

Lafrance H, Guillot M, Germain L, Auger FA (1995) A method for the evaluation of tensile properties of skin equivalents. *Medical Engineering and Physics* **17**(7)**:** 537-543

Lai WM, Mow VC (1980) Drag-induced compression of articular cartilage during a permeation experiment. *Biorheology* **17**(1-2)**:** 111-123

Langer R, Vacanti JP (1993) Tissue engineering. *Science* **260**(5110)**:** 920-926

Lantz MS, Ciborowski P (1994) Zymographic techniques for detection and characterization of microbial proteases. *Methods in Enzymology* **235:** 563-594

Laurent GJ (1987) Dynamic state of collagen: pathways of collagen degradation in vivo and their possible role in regulation of collagen mass. *American Journal of Physiology - Cell Physiology* **252**(1)**:** 1-9

Lee CH, Singla A, Lee Y (2001a) Biomedical applications of collagen. *International Journal of Pharmaceutics* **221**(1–2)**:** 1-22

Lee CR, Grodzinsky AJ, Spector M (2001b) The effects of cross-linking of collagen-glycosaminoglycan scaffolds on compressive stiffness, chondrocyte-mediated contraction, proliferation and biosynthesis. *Biomaterials* **22**(23)**:** 3145-3154

Lee KY, Alsberg E, Mooney DJ (2001c) Degradable and injectable poly(aldehyde guluronate) hydrogels for bone tissue engineering. *Journal of Biomedical Materials Research* **56**(2)**:** 228-233

Lee KY, Mooney DJ (2001) Hydrogels for tissue engineering. *Chemical Reviews* **101**(7)**:** 1869-1880

Liabakk N-B, Talbot I, Smith RA, Wilkinson K, Balkwill F (1996) Matrix Metalloprotease 2 (MMP-2) and Matrix Metalloprotease 9 (MMP-9) Type IV Collagenases in Colorectal Cancer. *Cancer Research* **56**(1)**:** 190-196

Lijnen PL, Petrov V, Rumilla K, Fagard R (2002) Stimulation of collagen gel contraction by angiotensin II and III in cardiac fibroblasts. *Journal of the Renin-Angiotensin-Aldosterone System* **3**(3)**:** 160-166

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry* **193**(1)**:** 265-275

S.J. MacGregor, J.G. Anderson, M. Maclean, G.A Woolsey, M. H. Grant, J. R. Beveridge, S. Griffiths, S. Smith, L. Murdoch and I. Timoshkin (2008) Developments and applications in Electronic Sterilisation Technologies. *Proceedings of the 17th International Conference on Gas Discharges and Their Applications*: 1-8

Mandl I, (1982) Bacterial collagenases and their clinical applications. *Arzneimittel-Forschung* **32**(10a)**:** 1381-1384

Mandl I, Maclennan JD, Howes EL (1953) Isolation and characterization of proteinase and collagenase from Cl. histolyticum. *Journal of Clinical Investigation* **32**(12)**:** 1323-1329

Matsuda K, Suzuki S, Isshiki N, Yoshioka K, Okada T, Ikada Y (1990) Influence of glycosaminoglycans on the collagen sponge component of a bilayer artificial skin. *Biomaterials* **11**(5)**:** 351-355

McPherson JM, Sawamura SJ, Conti A (1986) Preparation of [3H] Collagen for studies of the biologic fate of xenogenic collagen implants in vivo. *Journal of Investigative Dermatology* **86**(6)**:** 673-677

Meyvis TKL, De Smedt SC, Demeester J, Hennink WE (1999) Rheological monitoring of long-term degrading polymer hydrogels. *Journal of Rheology* **43**(4)**:** 933-950

Miles AP, Saul A (2005) Quantifying recombinant proteins and their degradation products using SDS-PAGE and scanning laser densitometry. *Methods in Molecular Biology* **308:** 349-56

Mookhtiar KA, Van Wart HE (1992) Clostridium histolyticum collagenases: a new look at some old enzymes. *Matrix Supplement* **1:** 116-26

Moore S, Stein WH (1948) Photometric ninhydrin method for use in the chromatography of amino acids. *Journal of Biological Chemistry* **176**(1)**:** 367-388

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods* **65**(1-2)**:** 55-63

Mow VC, Holmes MH, Michael Lai W (1984) Fluid transport and mechanical properties of articular cartilage: A review. *Journal of Biomechanics* **17**(5)**:** 377-394

Mow VC, Kuei SC, Lai WM, Armstrong CG (1980) Biphasic creep and stress relaxation of articular cartilage in compression: Theory and experiments. *Journal of Biomechanical Engineering* **102**(1)**:** 73-84

Mudera VC, Pleass R, Eastwood M, Tarnuzzer R, Schultz G, Khaw P, McGrouther DA, Brown RA (2000) Molecular responses of human dermal fibroblasts to dual cues: Contact guidance and mechanical load. *Cell Motility and the Cytoskeleton* **45**(1)**:** 1-9

Murphy G, Cartwright EC, Sellers A, Reynolds JJ (1977) The detection and characterization of collagenase inhibitors from rabbit tissues in culture. *Biochimica et Biophysica Acta (BBA) - Enzymology* **483**(2)**:** 493-498

Muyonga JH, Cole CGB, Duodu KG (2004) Extraction and physico-chemical characterisation of Nile perch (Lates niloticus) skin and bone gelatin. *Food Hydrocolloids* **18**(4)**:** 581-592

Nagase H, Fushimi K (2008) Elucidating the function of non catalytic domains of collagenases and aggrecanases. *Connective Tissue Research* **49**(3-4)**:** 169-174

Nagase H, Visse R, Murphy G (2006) Structure and function of matrix metalloproteinases and TIMPs. *Cardiovascular Research* **69**(3)**:** 562-573

Nagase H, Woessner JF (1999) Matrix Metalloproteinases. *Journal of Biological Chemistry* **274**(31)**:** 21491-21494

Nazhat SN, Abou Neel EA, Kidane A, Ahmed I, Hope C, Kershaw M, Lee PD, Stride E, Saffari N, Knowles JC, Brown RA (2006) Controlled microchannelling in dense collagen scaffolds by soluble phosphate glass fibers. *Biomacromolecules* **8**(2)**:** 543-551

Nelson AR, Fingleton B, Rothenberg ML, Matrisian LM (2000) Matrix metalloproteinases: biologic activity and clinical implications. *Journal of Clinical Oncology* **18**(5)**:** 1135-1135

Neuberger A, Perrone JC, Slack HG (1951) The relative metabolic inertia of tendon collagen in the rat. *Biochemical Journal* **49**(2)**:** 199-204

Neuberger A, Slack HG (1953) The metabolism of collagen from liver, bone, skin and tendon in the normal rat. *Biochemical Journal* **53**(1)**:** 47-52

Nissen R, Cardinale GJ, Udenfriend S (1978) Increased turnover of arterial collagen in hypertensive rats. *Proceedings of the National Academy of Sciences* **75**(1)**:** 451-453

Olson JL, Atala A, Yoo JJ (2011) Tissue engineering: current strategies and future directions. *Chonnam Medical Journal* **47**(1)**:** 1-13

Ornstein L (1964) Disc electrophoresis-I background and theory. *Annals of the New York Academy of Sciences* **121**(2)**:** 321-349

Osborne C, Barbenel J, Smith D, Savakis M, Grant M (1998) Investigation into the tensile properties of collagen/chondroitin-6-sulphate gels: the effect of crosslinking agents and diamines. *Medical and Biological Engineering and Computing* **36**(1)**:** 129-134

Overall CM, López-Otín C (2002) Strategies for MMP inhibition in cancer: innovations for the post-trial era. *Nature Reviews Cancer* **2**(9)**:** 657-672

Pal K, Banthia AK, Majumdar DK (2009) Polymeric hydrogels: Characterization and biomedical applications. *Designed Monomers & Polymers* **12**(3)**:** 197-220

Park S-N, Park J-C, Kim HO, Song MJ, Suh H (2002) Characterization of porous collagen/hyaluronic acid scaffold modified by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide cross-linking. *Biomaterials* **23**(4)**:** 1205-1212

Persidsky MD, Baillie GS (1977) Fluorometric test of cell membrane integrity. *Cryobiology* **14**(3)**:** 322-331

Petersen OW, Rønnov-Jessen L, Howlett AR, Bissell MJ (1992) Interaction with basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant human breast epithelial cells. *Proceedings of the National Academy of Sciences* **89**(19)**:** 9064-9068

Prajapati RT, Chavally-Mis B, Herbage D, Eastwood M, Brown RA (2000) Mechanical loading regulates protease production by fibroblasts in three-dimensional collagen substrates. *Wound Repair and Regeneration* **8**(3)**:** 226-237

Prontera C, Mariani B, Rossi C, Poggi A, Rotilio D (1999) Inhibition of gelatinase A (MMP-2) by batimastat and captopril reduces tumor growth and lung metastases in mice bearing Lewis lung carcinoma. *International Journal of Cancer* **81**(5)**:** 761-766

Périé D, Korda D, Iatridis JC (2005) Confined compression experiments on bovine nucleus pulposus and annulus fibrosus: sensitivity of the experiment in the determination of compressive modulus and hydraulic permeability. *Journal of Biomechanics* **38**(11)**:** 2164-2171

Périé D, Maclean J, Owen J, Iatridis J (2006) Correlating material properties with tissue composition in enzymatically digested bovine annulus fibrosus and nucleus pulposus tissue. *Annals of Biomedical Engineering* **34**(5)**:** 769-777

Ranta-Eskola AJ (1979) Use of the hydraulic bulge test in biaxial tensile testing. *International Journal of Mechanical Sciences* **21**(8)**:** 457-465

Reinhardt D, Sigusch HH, Henβe J, Tyagi SC, Körfer R, Figulla HR (2002) Cardiac remodelling in end stage heart failure: upregulation of matrix metalloproteinase (MMP) irrespective of the underlying disease, and evidence for a direct inhibitory effect of ACE inhibitors on MMP. *Heart* **88**(5)**:** 525-530

Reynolds JA, Tanford C (1970) Binding of dodecyl sulfate to proteins at high binding ratios. Possible implications for the state of proteins in biological membranes. *Proceedings of the National Academy of Sciences* **66**(3)**:** 1002-1007

Riches PE (2011) Sensitivity analysis of permeability parameters of bovine nucleus pulposus obtained through inverse fitting of the nonlinear biphasic equation: effect of sampling strategy. *Computer Methods in Biomechanics and Biomedical Engineering***:** 1-8

Riches PE, McNally DS (2005) A one-dimensional theoretical prediction of the effect of reduced end-plate permeability on the mechanics of the intervertebral disc. *Proceedings of the Institution of Mechanical Engineers, Part H: Journal of Engineering in Medicine* **219**(5)**:** 329-335

Říhová B (2000) Immunocompatibility and biocompatibility of cell delivery systems. *Advanced Drug Delivery Reviews* **42**(1–2)**:** 65-80

Roberts S, Urban JPG, Evans H, Eisenstein SM (1996) Transport properties of the human cartilage endplate in relation to its composition and calcification. *Spine* **21**(4)**:** 415-420

Robinson KA, Li J, Mathison M, Redkar A, Cui J, Chronos NAF, Matheny RG, Badylak SF (2005) Extracellular matrix scaffold for cardiac repair. *Circulation* **112**(9)**:** 135-143

Roeder BA, Kokini K, Sturgis JE, Robinson JP, Voytik-Harbin SL (2002) Tensile mechanical properties of three-dimensional type I collagen extracellular matrices with varied microstructure. *Journal of Biomechanical Engineering* **124**(2)**:** 214-222

Rosso F, Giordano A, Barbarisi M, Barbarisi A (2004) From Cell–ECM interactions to tissue engineering. *Journal of Cellular Physiology* **199**(2)**:** 174-180

Royce PM, Kato T, Ohsaki K-I, Miura A (1995) The enhancement of cellular infiltration and vascularisation of a collagenous dermal implant in the rat by platelet-derived growth factor BB. *Journal of Dermatological Science* **10**(1)**:** 42-52

Rucklidge GJ, Milne G, McGaw BA, Milne E, Robins SP (1992) Turnover rates of different collagen types measured by isotope ratio mass spectrometry. *Biochimica et Biophysica Acta (BBA) - General Subjects* **1156**(1)**:** 57-61

Saddiq ZA, Barbenel JC, Grant MH (2009) The mechanical strength of collagen gels containing glycosaminoglycans and populated with fibroblasts. *Journal of Biomedical Materials Research* **89A**(3)**:** 697-706

SANTYL® (2011) (*www.santyl.com*). TX, USA: Healthpoint® Biotherapeutics

Shapiro AL, Vinuela E, Maizel JV, Jr. (1967) Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. *Biochemical and Biophysical Research Communications* **28**(5)**:** 815-20

Shaw T, Nixon JS, Bottomley KM (2000) Metalloproteinase inhibitors: new opportunities for the treatment of rheumatoid arthritis and osteoarthritis. *Expert Opinion on Investigational Drugs* **9**(7)**:** 1469-1478

Shen Zhilei L, Kahn H, Ballarini R, Eppell Steven J (2011) Viscoelastic properties of isolated collagen fibrils. *Biophysical Journal* **100**(12)**:** 3008-3015

Sheridan RL, Hegarty M, Tompkins RG, Burke JF (1994) Artificial skin in massive burns — results to ten years. *European Journal of Plastic Surgery* **17**(2)**:** 91-93

Shoulders MD, Raines RT (2009) Collagen structure and stability. *Annual Review of Biochemistry* **78:** 929-958

Silva EA, Mooney DJ (2004) Synthetic extracellular matrices for tissue engineering and regeneration. *Current Topics in Developmental Biology*, **64:** 181-205

Snoek-van Beurden PA, Von den Hoff JW (2005) Zymographic techniques for the analysis of matrix metalloproteinases and their inhibitors. *Biotechniques* **38:** 73-83

Sorbi D, Fadly M, Hicks R, Alexander S, Arbeit L (1993) Captopril inhibits the 72 kDa and 92 kDa matrix metalloproteinases. *Kidney International* **44**(6)**:** 1266-1272

Soulhat J, Buschmann MD, Shirazi-Adl A (1999) A fibril-network-reinforced biphasic model of cartilage in unconfined compression. *Journal of Biomechanical Engineering* **121**(3)**:** 340-347

Stammen JA, Williams S, Ku DN, Guldberg RE (2001) Mechanical properties of a novel PVA hydrogel in shear and unconfined compression. *Biomaterials* **22**(8)**:** 799-806

Sternlicht MD, Werb Z (2001) How matrix metalloproteinases regulate cell behavior. *Annual Review of Cel andl Developmental Biology* **17:** 463-516

Suh JK, DiSilvestro MR (1999) Biphasic poroviscoelastic behavior of hydrated biological soft tissue. *Journal of Applied Mechanics* **66**(2)**:** 528-535

Tibbitt MW, Anseth KS (2009) Hydrogels as extracellular matrix mimics for 3D cell culture. *Biotechnology and Bioengineering* **103**(4)**:** 655-663

Todaro GJ, Green H (1963) Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. *Journal of Cell Biology* **17:** 299-313

Troeberg L, Nagase H (2001) Zymography of metalloproteinases. *Current Protocols in Protein Science*, John Wiley & Sons, Inc. Section 21.15

Urban JP, Holm S, Maroudas A (1978) Diffusion of small solutes into the intervertebral disc: as in vivo study. *Biorheology* **15**(3-4)**:** 203-21

Visse R, Nagase H (2003) Matrix metalloproteinases and tissue inhibitors of metalloproteinases. *Circulation Research* **92**(8)**:** 827-839

Wagenseil JE, Wakatsuki T, Okamoto RJ, Zahalak GI, Elson EL (2003) One-dimensional viscoelastic behavior of fibroblast populated collagen matrices. *Journal of Biomechanical Engineering* **125**(5)**:** 719-725

Wakatsuki T, Kolodney MS, Zahalak GI, Elson EL (2000) Cell mechanics studied by a reconstituted model tissue. *Biophysical Journal* **79**(5)**:** 2353-2368

Waldman SD, Couto DC, Grynpas MD, Pilliar RM, Kandel RA (2006) A single application of cyclic loading can accelerate matrix deposition and enhance the properties of tissue-engineered cartilage. *Osteoarthritis and Cartilage* **14**(4)**:** 323-330

Wallace DG, Rosenblatt J (2003) Collagen gel systems for sustained delivery and tissue engineering. *Advanced Drug Delivery Reviews* **55**(12)**:** 1631-1649

Weber K, Osborn M (1969) The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *Journal of Biological Chemistry* **244**(16)**:** 4406-4412

Williams RN, Parsons SL, Morris TM, Rowlands BJ, Watson SA (2005) Inhibition of matrix metalloproteinase activity and growth of gastric adenocarcinoma cells by an angiotensin converting enzyme inhibitor in in vitro and murine models. *European Journal of Surgical Oncology (EJSO)* **31**(9)**:** 1042-1050

Wojtowicz-Praga SM, Dickson RB, Hawkins MJ (1997) Matrix metalloproteinase inhibitors. *Investigational New Drugs* **15**(1)**:** 61-75

Worthington K, Worthington V (2011) Worthington Enzyme Manual (*http://www.worthington-biochem.com/pap/default.html*). NJ, USA: Worthington Biochemical Corporation

Wulf E, Deboben A, Bautz FA, Faulstich H, Wieland T (1979) Fluorescent phallotoxin, a tool for the visualization of cellular actin. *Proceedings of the National Academy of Sciences* **76**(9)**:** 4498-4502

Yamamoto D, Takai S, Jin D, Inagaki S, Tanaka K, Miyazaki M (2007a) Molecular mechanism of imidapril for cardiovascular protection via inhibition of MMP-9. *Journal of Molecular and Cellular Cardiology* **43**(6)**:** 670-676

Yamamoto D, Takai S, Miyazaki M (2007b) Prediction of interaction mode between a typical ACE inhibitor and MMP-9 active site. *Biochemical and Biophysical Research Communications* **354**(4)**:** 981-984

Yamamoto D, Takai S, Miyazaki M (2008) Inhibitory profiles of captopril on matrix metalloproteinase-9 activity. *European Journal of Pharmacology* **588**(2–3)**:** 277-279

Yang S, Leong KF, Du Z, Chua CK (2001) The design of scaffolds for use in tissue engineering. Part I. Traditional factors. *Tissue Eng* **7**(6)**:** 679-89

Yannas IV (1990) Biologically active analogues of the extracellular matrix: artificial skin and nerves. *Angewandte Chemie International Edition in English* **29**(1)**:** 20-35

Yannas IV, Burke JF (1980) Design of an artificial skin. I. Basic design principles. *Journal of Biomedical Materials Research* **14**(1)**:** 65-81

Yoshii S, Oka M (2001) Collagen filaments as a scaffold for nerve regeneration. *Journal of Biomedical Materials Research* **56**(3)**:** 400-405

Yoshii S, Oka M, Shima M, Taniguchi A, Akagi M (2003) Bridging a 30-mm nerve defect using collagen filaments. *Journal of Biomedical Materials Research Part A* **67A**(2)**:** 467-474

Zucker S, Lysik RM, Zarrabi MH, Moll U (1993) Mr 92,000 Type IV collagenase is increased in plasma of patients with colon cancer and breast cancer. *Cancer Research* **53**(1)**:** 140-146