Investigating the Biophysical Properties of Ageing of Collagenous Tissue at the Nanoscale

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I, Tarek Ahmed confirm that the work presented in this thesis is my own.
Where information has been derived from other sources, I confirm that this has been indicated in the thesis.
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Abstract

Collagen is the most abundant protein in the human body and there are a number of changes that occur to collagen as we age, the main being the accumulation of adventitious advanced glycation end products (AGEs). These can be in the form of covalent cross-links forming between residues within neighbouring collagen molecules. Glucosepane is the most common AGE cross-link found in collagenous tissue and like other AGEs, its impact on the collagen matrix at the nanoscale is not fully understood. This thesis investigates the biophysical properties of collagenous tissue as a function of ageing due to AGE accumulation, in particular Glucosepane. The study identifies nanoscale markers of ageing (morphological and mechanical) and assesses the presence of these markers in various human tissues. The study takes an in-vitro approach to develop glycated tissue models, mimicking the ageing of tissue in the lab. Ex-vivo collagenous tissue samples from donors spanning a variety of ages are also assessed to identify the presence of the markers discovered. The study identifies unique features of the properties of collagen at the nanoscale spurred by age and accumulation of AGEs, including changes in collagen fibrillar structure as well as fibrillar mechanical properties. This thesis proposes a novel collagen-water interaction mechanism which has significant effects on the biophysical properties of collagen as AGEs accumulate.
Impact statement

This research project was motivated by applying tools and techniques in nanotechnology to explore various physiological processes associated with ageing. In doing so, there have been a number of developments in understanding which are applicable to wider topics outside of ageing and this project, in both research and commercial settings.

Firstly, this research project developed a series of techniques for nanodiagnostics of connective tissue, for both in-vitro and ex-vivo samples. This included sample preparation, characterisation and analysis. While the use of non-invasive AFM based nanodiagnostics is not currently used conventionally, this study highlights its potential and validity as a technique for use in a healthcare context. The techniques developed in this study may be directly applied to a wide range of disease indications, spanning connective tissue disorders. This includes various forms of fibrosis, scleroderma, Marfan syndrome, Ehler-Danlos syndrome, and osteogenesis imperfecta as examples. Upon further study to identify nanoscale biomarkers of mentioned diseases, their onset and progression can be identified to a greater degree of sensitivity, potentially improving diagnosis rates and characterisation of progression.

Secondly, this study produced tissue engineering extracellular matrix which more closely matched in-vivo tissue due to cross-linking of the collagen matrix. The future of implants is regenerative medicine. Regenerative medicine requires scaffolds to be biomimetic and so implanting scaffolds which have undergone the "ageing process" improves biomimeticity. Collagen is used ubiquitously in tissue engineering scaffolds, therefore the use of in-vitro glycation to improve scaffold biomimeticity is profound. Products which utilise glycation in scaffold formation are one step closer to mimicking human tissue.
Thirdly, the impact of advanced glycation end product cross-linking is important in both an ageing as well as diabetes context. While there exist several techniques to diagnose diabetes (e.g. blood-glucose testing, HbA1c testing), the techniques developed in this study can indicate the severity of the impact of diabetes by measuring the extent of physiological changes in connective tissue for various organs.

While the applications of this research are comprehensive, the techniques developed in this study require further refinement to use in the contexts mentioned. This research however demonstrates the potential of AFM nanodiagnostics as well as newly identified biophysical properties of collagen as the plinth for an array of products serving multiple therapeutic areas.
3.17 Scanning electron microscopy .......................................................................................... 79
3.18 Transmission electron microscopy ................................................................................. 79
3.19 Sampling .................................................................................................................................... 80
3.20 Statistical analysis .................................................................................................................. 81
3.21 Code ............................................................................................................................................. 82
4 Development of Studies ................................................................................................................... 85
4.1 Aim of the chapter ...................................................................................................................... 85
4.2 Developing an in-vitro model ................................................................................................ 86
  4.2.1 In-vitro methods .................................................................................................................... 86
  4.2.2 Finding the right pH ............................................................................................................ 87
  4.2.3 Neutralisation of collagen gels .......................................................................................... 89
  4.2.4 Glycating agents for the in-vitro glycated tissue models ............................................ 90
  4.2.5 Concentration of glycating agents ................................................................................. 91
  4.2.6 Concentration assay for glycating agents .................................................................... 92
  4.2.7 Nanoscale morphology of native collagen .................................................................... 95
  4.2.8 Indentation load ................................................................................................................ 96
4.3 Results and Discussion ............................................................................................................. 97
  4.3.1 Identifying morphological markers of ageing in in-vitro glycated tissue models 97
  ........................................................................................................................................................ 106
  4.3.2 Summary of morphological markers ............................................................................. 106
4.3.3 Identifying nanomechanical markers of glycated tissue models ........... 107

4.4 Key findings ............................................................................................................................... 120

4.4.1 Nanomechanics .................................................................................................................... 120

4.4.2 Understanding the control in-vitro tissue model ................................................................. 120

4.4.3 Hydrogen bonding revisited ............................................................................................... 121

4.4.4 The effect of hydrogen bonding ............................................................................................ 122

4.4.5 Decreasing elastic modulus shows a reduction in fibril density ........................................ 122

4.4.6 Decreasing contour length shows a reduction in collagen molecule affinity ...................... 123

4.4.7 Differences in glycation properties across the different models ........................................ 125

4.4.8 The importance of AGE stability ......................................................................................... 127

4.4.9 Proposed mechanism: AGEs provide an additional form of collagen-hydration interaction, increasing fibril hydration and decreasing fibril density ... 128

4.4.10 Wider studies of fibril hydration in literature ................................................................. 133

4.5 Conclusions ............................................................................................................................ 133

5 Investigation of Ageing of Dermis ............................................................................................. 135

5.1 Introduction ............................................................................................................................... 135

5.2 Preparing samples for application of techniques ................................................................... 135

5.2.1 Cryosectioning tissue ......................................................................................................... 136

5.2.2 Characterising skin ............................................................................................................ 138

5.2.3 Non collagenous components of the dermis ....................................................................... 139
5.2.4 Microscale to nanoscale analysis with electron microscopy – the pitfalls

5.3 Results .................................................................................................................. 143

5.3.1 Histology .......................................................................................................... 143

5.3.2 Microscale morphology .................................................................................... 144

5.3.3 Nanoscale morphology ...................................................................................... 146

5.3.4 Fibril swelling – an initial study ....................................................................... 149

5.3.5 Nanoindentation ................................................................................................ 150

5.4 Key findings .......................................................................................................... 159

5.4.1 Morphological markers ..................................................................................... 159

5.4.2 Elastic modulus of individual fibrils decreases with age ................................. 160

5.4.3 Age dependent reduction is not apparent in hydrated sample ....................... 162

5.4.4 Proposed hypothesis - Increased hydration is an evolutionary response to loss of other hydrophilic components ................................................................. 163

5.5 Conclusions ......................................................................................................... 164

6 Investigation of ageing of tendon .......................................................................... 165

6.1 Introduction ........................................................................................................... 165

6.2 Preparing tendon for application of techniques .................................................. 166

6.2.1 Cryosectioning without cryo-embedding medium ......................................... 168

6.2.2 Rinsing of sections ............................................................................................ 169

6.3 Results .................................................................................................................. 170

6.3.1 Histology .......................................................................................................... 170
Abbreviations

µCT – micro-computerised tomography
AFM – atomic force microscope
AGEs - Advanced Glycation End products
ANOVA – analysis of variance
CML – Carboxymethyllysine
COL11A1 - Collagen, type XI, alpha 1
COL11A2 - Collagen, type XI, alpha 2
COL1A1 - Collagen, type I, alpha 1
COL1A2 - Collagen, type I, alpha 2
COL2A1 - Collagen, type II, alpha 1
COL3A1 - Collagen, type III, alpha 1
COL5A1 - Collagen, type V, alpha 1
COL5A2 - Collagen, type V, alpha 2
COL5A3 - Collagen, type V, alpha 3
deH-DHNLNL - dehydro-dihydroxylysinonorleucine
deH-HLNL - dehydro-hydroxylysinonorleucine
deH-LNL - dehydro-lysinonorleucine
DNA - Deoxyribonucleic acid
DOGDIC - deoxyglucosone-derived imidazolium cross-link
DOLD - deoxyglucosone lysine dimer
DPD – Deoxy-pyridinoline
DPL – Deoxy-pyrrololine
DSC – differential scanning calorimetry
ECM – Extracellular matrix
F-D – force displacement
FEG – field emission gun
FJC – freely jointed chain
GAG - Glycosaminoglycan
GAG – glycosaminoglycan
G-H – glyoxal based hydroimidazolimine
GODIC - glyoxal imidazolimine cross-link
GOLA - glyoxal lysine amide
GOLD – glyoxal derived lysine dimer
H&E – Haemotoxin and eosin
HLA - Hyaluronic acid
HLKLN - hydroxylysino-5-kenonorleucine
LKLN - lysino-5-ketonorleucine
LOX – Lysyl oxidase
MD – Molecular dynamic
MEM – minimum essential medium
MG-H – methylglyoxal based hydroimidazonones
MODIC - methylglyoxal imidazonlimine cross-link;
MOLD - methylglyoxal derived lysine dimer
MRI – magnetic resonance imaging
OCT – Optical coherence tomography
PBS – phosphate buffered saline
PC – plastically compressed
PR – pico sirius
PYD – Pyridinoline

PYL – Pyrrololine

RAFT – Real Architecture for 3D Tissues

RAGE - receptor for advanced glycation end products

REC ref – Research Ethics Committee reference

ROS – Reactive oxygen species

SEM – scanning electron microscope

SMFS – single molecule force spectroscopy

TEM – transmission electron microscope

UHQ – ultra high quality

WLC – worm-like chain
1 Introduction

1.1 The problem

Understanding how the human body ages is complex but timely, as the proportion of older population increases. In a recent report by the UK House of Lords (Lumpkin and Caterina) it was suggested that children born after 2010 have life expectancies of 96 and 93 years girls and boys, respectively (Sawicki, Lewis et al. 2009). It is therefore essential to understand the nature and impact of the ageing process on organs, as physiological and functional characteristics of these organs change dramatically.

1.2 Mechanisms of ageing

Ageing is a process that we are all familiar with, however it is a complex phenomenon of which there is much yet to be understood. Ageing can be described as the increasing failure with time of organs to maintain their function due to the loss of tissue homeostasis and regeneration (Liu and Rando 2011). This results in the sustained loss in the ability of an organism to counter disease and withstand environmental threats due to impaired physical structure.

Ageing in itself is not defined by a singular process, with a variety of different pathways leading to the phenomenon described above. There is certainly an effect of genetics on the lifespan of individuals, including specific genes with impact on longevity, however the resulting effect on lifespan can vary significantly. The various mechanisms of ageing at the molecular level include the alteration of proteins and accumulation of waste (Terman and Brunk 2004), DNA damage and somatic mutation (Promislow 1994), the loss of telomeres with cell division and the build-up of mitochondrial DNA. It is likely that the
combined effect of the various mechanisms contribute to cell damage which in turn results in undesirable apoptotic behaviour, cascading into senescence (Kirkwood, Boys et al. 2003).

This study investigates in detail one of the mechanisms – alteration of protein due to accumulation of waste. In particular the alteration of collagen due to the accumulation of Advanced Glycation End products (AGEs).

1.3 Collagen

1.3.1 Why collagen?

Collagen is the most abundant protein found in mammals and constitutes a major component of connective tissues, forming 20-25% of body protein (Vuorio and De Crombrugghe 1990, Di Lullo, Sweeney et al. 2002, Stefanovic 2013). Understanding age related changes in the biophysical properties is important in developing knowledge of the impact of ageing on the extracellular matrix of connective tissue at the nanoscale. It is yet unclear what occurs to the structure of collagen at this scale and due to the importance of extracellular matrix properties in providing functional properties to the surrounding tissue it is becoming increasingly apparent that a greater understanding of the ageing process is required. With the development of new tools and techniques in nanotechnology, it is now possible to investigate ageing at this fundamental scale.

1.3.2 Roles and function of collagenous tissue

Collagen has a primary role in the extracellular matrix of various tissues providing structure and mechanical support. Not only does the extracellular matrix physically keep
the shape and structure of tissues and organs intact, it also provides a framework for cells to grow, allowing cell adhesion and cell signalling to take place. Collagen also has a role in the storage and delivery of growth factors and cytokines, affecting important cellular interactions. The importance of collagen is therefore paramount in providing the biomechanical and biochemical environment necessary for the functioning of various tissues. This study investigates the impact of ageing on two connective tissues in particular, tendon and dermis.

1.3.3 Collagen molecular structure

Collagen is composed of three polypeptide chains (Ramachandran and Kartha 1955) which are wrapped around each other to form a triple helix. The polypeptide chains are composed mostly of the amino acid residues Glycine – X – Y where (X and Y are often Proline and Hydroxyproline) and form an alpha chain like (they are not strictly alpha chains and do not form common alpha helix higher order structure) structure consisting of approximately a Glycine as every third residue. The polypeptide alpha chains consist of approximately 1000 residues and have left handed helical twist making them differ from the conventional alpha chain, which contain right handed symmetry. The collagen triple helix has 3 amino acids corresponding to one turn in the helix (however this can vary across the chain and with collagen type) (Kramer, Bella et al. 1999, Boudko, Engel et al. 2008).

The alpha chains wrap around each other to form a molecule 297 nm long and 1.5 nm wide with a right handed triple helix structure 2005). The 3 alpha chains are held together by hydrogen bonds between NH groups of Glycine residues and C=O bonds from neighbouring alpha chains and lysine derived inter and intra molecular cross-links also
add to give the triple helix stability. The presence of Glycine is crucial for the triple helical structure of collagen molecules, it is the smallest amino acid and allows the tight wrapping of the 3 chains. Collagen based deformities such as Osteogenesis Imperfecta can be a result of substitution or mutations of Glycine (Cole, Chan et al. 1996). The residues Proline and Hydroxyproline also support the cyclical structure of collagen, preventing excessive rotation of the polypeptide chain (Beck, Chan et al. 2000).

1.3.4 Collagen types

There are a number of different collagen types (Table 1). Variety in amino acid sequences gives rise to the presence of α1, α2 and α3 chains. Combinations of these within the triple helix give rise to a variety of collagen types where 3 identical alpha chains form homotrimers and 2 or 3 different alpha chains form heterotrimers. There have been 28 different collagen types found in the human body (Fratzl 2008). These are separated into 2 main groups – fibrillar and non fibrillar collagen.

1.3.4.1 Fibrillar collagen

Fibrillar collagen is formed by collagen molecules aggregating together to form fibrils. Collagen molecules aggregate in an entropy driven process to form pentafibrils. These then self-assemble to form larger scale fibrillar structures eventually forming fibres with dimensions dependent upon their location in the body and the forces they are subjected to. The most common collagen found in humans is type I collagen. This is a fibrillar collagen consists of two α1 chains (alpha1(I)) and one α2 chain (alpha2(I)). Type I collagen is found in skin, bone, tendon, lung, cornea and vascular tissue, often as a major component of the extracellular matrix.
Other fibrillar collagen types include type II, type III, type V and type XI (table 1.1). Type II collagen is found in cartilage and vitreous humour and consists of three α1 chains (alpha1(II)) wrapped in a triple helical homotrimer. Type III collagen is found in embryonic tissue, skin, lung and vasculature, is again composed of three α1 chains (lpha1(III)). Type V collagen is found in the cornea as well as in regions consisting of collagen type I. Type V collagen can be composed of three different alpha chains (alpha1(V), alpha2(V), alpha3(V)). Type XI collagen is found as a small component of cartilage and is composed of three different alpha chains forming a heterotrimer. However the α3 chain in this case is the same as a modified alpha1(II) chain.

Collagen molecules from fibrillar collagens share the long triple helical region with collagen types I, II and III also containing non helical telopeptides at either ends of the helical alpha chains (N and C termini). These telopeptides are approximately 20 residues long, giving the molecule solubility.

<table>
<thead>
<tr>
<th>Type</th>
<th>Molecule Composition</th>
<th>Structural Features</th>
<th>Representative Tissues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrillar Collagens</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>α1(I)[2α2(I)]</td>
<td>300-nm-long fibrils</td>
<td>Skin, tendon, bone, ligaments, dentin, interstitial tissues</td>
</tr>
<tr>
<td>II</td>
<td>α1(II)β3</td>
<td>300-nm-long fibrils</td>
<td>Cartilage, vitreous humor</td>
</tr>
<tr>
<td>III</td>
<td>α1(III)β3</td>
<td>300-nm-long fibrils</td>
<td>Skin, muscle, blood vessels</td>
</tr>
<tr>
<td></td>
<td></td>
<td>globular N-terminal</td>
<td>Similar to type I; also cell cultures, fetal tissues</td>
</tr>
<tr>
<td></td>
<td></td>
<td>domain; often with</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>type I</td>
<td></td>
</tr>
<tr>
<td>Fibril-Associated Collagens</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>[α1(VI)][α2(VI)]</td>
<td>Lateral association</td>
<td>Most interstitial tissues</td>
</tr>
<tr>
<td></td>
<td></td>
<td>with type I</td>
<td></td>
</tr>
<tr>
<td>IX</td>
<td>[α1(IX)][α2(IX)]</td>
<td>Lateral association</td>
<td>Cartilage, vitreous humor;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>with type II</td>
<td></td>
</tr>
<tr>
<td>Sheet-Forming Collagens</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>[α1(V)][α2(V)]</td>
<td>Two-dimensional network</td>
<td>All basal laminae</td>
</tr>
</tbody>
</table>

Table 1.1: The most common types of collagen and their locations within the body (Mayne and Burgeson 1987)
1.3.4.2 Non fibrillar collagen

There are a number of collagens that form non-fibrillar supramolecular structures such as membranes and filaments (Figure 1.1).

There are 3 distinct groups of non fibrillar collagens. These include;

- Network collagens: Network collagens include long type-IV and short type-VIII and X collagen molecules, which are approximately 400 nm long and arranged in a mesh like network.
- Filament collagens: Type-VI collagen molecules pack adjacently and repeating axially every 100 nm.
- Fibril associated collagens: These collagens are located near other fibrillar collagens however don't form fibrils themselves. Examples of this are type-XII and type-XIV collagens which can be found near fibrillar collagen type-I (Sugrue, Gordon et al. 1989).

Non fibrillar collagens are not as abundant as fibrillar collagens however still have an important role and function in the extracellular matrix of various tissues.

![Figure 1.1: Different collagen types (Bailey, Paul et al. 1998)](image-url)
This study will investigate tissues comprising of mostly fibrillar type-1 collagen.

1.3.5 Biosynthesis

Collagen is produced in the body by many different cells such as endothelium cells, osteoblasts, odontoblasts, chondroblasts and fibroblasts (Langness and Undenfriend 1971). Fibroblasts are seen as the typical collagen producing cells as they are the most common cells found in connective tissue. The process of collagen biosynthesis involves a number of steps. Collagen, like all proteins is synthesised through genetic expression leading to transcription, translation and post translational modification (Crick 1970). An important note regarding collagen synthesis is the fact that it is not encoded by a single gene.

1.3.5.1 Genetic expression and transcription

The α1 and α2 chains in type I collagen are encoded by the COL1A1 and COL1A2 genes respectively. The three α1 chains (pro-alpha1(II)) producing type II collagen are encoded by the COL2A1 gene. Type III collagen is also composed of three α1 chains (pro-alpha1(III)) that are encoded by the COL3A1 gene. The three different alpha chains making up collagen type V (pro-alpha1(V), pro-alpha2(V), pro-alpha3(V)), are encoded by the COL5A1, COL5A2 and COL5A3 genes. Type XI collagen is found as a small component of cartilage and is composed of three different alpha chains forming a heterotrimer. However the α3 chain in this case is the same as a modified pro-alpha1(II) chain. The alpha chains are encoded by the COL11A1, COL11A2 and COL2A1 gene.
Once the genes are transcribed, mRNA migrates outside the nucleus into the cytoplasm where it can direct peptide chain synthesis to produce the selected amino acid sequence. This formation of an amino acid sequence produces pre pro-alpha chains. They are called alpha chains in this case due to their helical nature. They are also referred to “pre” pro-alpha chains due to the signal sequence at the beginning of the peptide (also known as a signal peptide). This is a short amino acid sequence found at the N-terminus of the chain that has been formed and allows the chains to enter the endoplasmic reticulum where the signal sequence is recognised by signal peptidase enzymes and cleaved and digested leaving the pro-alpha chain.

1.3.5.2 Post translational modification

The pro-alpha chains undergo a number of post translational modifications after being formed.

Hydroxylation

The pro-alpha chain encounters 2 enzymes (Prolyl hydroxylase and Lysyl Hydroxylase), which hydroxylate Proline and Lysine residues along the pro-alpha chain. Hydroxylation involves the addition of an OH (or Hydroxyl) group to the residue.

Glycosylation

Selected hydroxylysine residues undergo addition of galactose or galactose and glucose. Glycosylation involves the addition of a carbohydrate to a hydroxyl group and is catalysed under the presence of the enzymes galactosyltransferase (galactose addition) and glycosyltransferase (glucose addition). Glycosyltransferase adds glucose to hydroxyl groups which have already undergone galactose addition (Eyre, Paz et al. 1984). It is
important to note that this occurs on pro-alpha chains which have not yet been wound into collagen molecules (Pokidysheva, Zientek et al. 2013).

1.3.5.3 Formation of triple helix and secretion from cell

After the post translational modifications of pro-alpha chains have occurred, they intertwine with further pro-alpha chains to produce a triple helix. The alpha chains begin to entwine at the C-terminus of the alpha chains beginning to form a triple helix with non-helical regions at either end. These immature collagen molecules are known as procollagen molecules due to loose ends at both ends of the triple helix molecule (propeptides). It is important to note that hydrogen bond cross-linking of the pro-alpha chains is a crucial requirement for the formation of the triple helix. Once the procollagen molecule has been secreted out of the cell, at the edge of the cell membrane the procollagen is processed by enzymatic cleavage of the non-helical caps of the procollagen molecule found at the N and C-termini, leaving telopeptides.

1.3.6 Fibril formation

Once the fibroblast has secreted collagen molecules into the extracellular matrix and propeptides have been cleaved, fibrillogenesis occurs, creating supramolecular collagenous structures. Until the propeptides are cleaved by enzymes, the molecule is called a procollagen molecule. The propetide at the N-terminus (N-peptide) for procollagens I, II, III are fully cleaved leaving just a short N-telopeptide remaining at the terminus. The telopeptide at the C-terminus (C-peptide) for fibrillar collagens are removed during biosynthesis.
The necessary environment for the formation of structures in the form of fibrils from collagen molecules has been studied greatly in vitro (Cassel 1966, Williams, Gelman et al. 1978, Leikin, Rau et al. 1995, Jiang, Hörber et al. 2004, Jiang, Khairy et al. 2004, Li, Asadi et al. 2009). Fibril formation is an entropic process. This means collagen molecules spontaneously self-assemble in order to reach a more thermodynamically stable state.

1.3.6.1 Mechanisms of fibrillogenesis

There are two proposed mechanisms for achieving fibrillogenesis. One is through hydrophobic interactions resulting in collagen molecules reducing the surface exposure of hydrophobic residues found in the alpha chains. In order for this to occur, collagen molecules must aggregate and align in a way to minimise exposure of non-polar regions within the molecules, releasing water molecules into the surroundings and thus increasing entropy of the surroundings (Cassel 1966, Cooper 1970).

Another mechanism is collagen molecule aggregation due to hydrogen bonding (either direct peptide to peptide or mediated by water molecules). Some have interpreted release of water molecules to support the idea that water mediated hydrogen bonds are the major driving factor for fibrillogenesis rather than hydrophobic interactions (Leikin, Rau et al. 1994, Leikin, Rau et al. 1995, Kar, Amin et al. 2006). However, the dominance of this mechanism over hydrophobic interactions has not been shown by modelling which suggests that there is no significant dominance of one mechanism over the other with both playing an important role in fibrillogenesis (Streeter and de Leeuw 2011).

The dependence of fibrillogenesis on pH has also been reported, further supporting the theory that electrostatic interactions drive the process (Jiang, Hörber et al. 2004, Harris and Reiber 2007, Li, Asadi et al. 2009). Fibrillogenesis over a 2 day period can occur from pH 6.0 however the rate is significantly slow and only small diameter fibrils are formed.
Between pH 6.9-9.2 fibrils formed are of similar diameter, with the rate of formation significantly increasing as pH increases (Li, Asadi et al. 2009). As fibrillogenesis is an entropic process, there is a temperature dependency. Fibrils are able to form at temperatures lower than the physiological temperature, however it has been observed that this affects fibril diameter, with lower temperatures resulting in fibrils with larger diameters (Holmes et al. 1986).

There are three stages of fibril formation; initiation, linear growth and lateral fusion (Birk, Zycband et al. 1989, Kar, Amin et al. 2006, Kadler, Hill et al. 2008). Initially aggregation of collagen molecules is slow, with little slight overlapping of the N and C termini of two molecules occurring. This is followed by further overlapping of neighbouring molecules with the fibril growing in size both laterally and longitudinally (Birk, Zycband et al. 1989, Kadler, Hill et al. 2008).

1.3.6.2 D-Banding

When high resolution scanning electron microscopy was carried out by Schmitt in 1942, a periodic pattern could be observed running across the collagen fibrils (Schmitt 1942). This observation was later followed up by similar details being viewed using atomic force microscopy (Chernoff 1992, Gathercole, Miles et al. 1993).

This characteristic banding periodicity occurs transversely along the length of the fibril with a spacing of approximately 67 nm, D and is known as “D-banding”. D has been shown to be subject to change depending on conditions of fibrillogenesis, with temperature having a major influence on the banding pattern.

It is apparent that as fibrils aggregate, they arrange themselves in a particular pattern, which gives rise to the D-banding. Whether the N and C telopeptides are present in the collagen molecule can impact on the D-banding. There have been a number of models
proposed to describe the ultrastructure of fibrils. The conventionally accepted Hodge and Petruska model describes how at least 5 individual tropocollagen molecules aggregate forming pentafibrils (approximately 3.5nm in diameter). It specifically describes how the molecules are aligned over each other in a “staggered” pattern (shown in Figure 1.2), giving rise to regions where molecules in the adjacent row overlap at N and C terminals and regions where adjacent molecules in the same row leave a gap at N and C terminals.

Figure 1.2: Staggered arrangement pattern of collagen molecules (Petruska and Hodge 1964)

The overlap and gap regions produce differences in fibril density and topography along the pentafibril depending on the number of molecules are aligned over each other (Petruska and Hodge 1964).

However this is a theoretical 2D model, which failed to take into account slight twisting of collagen molecules and full spatial arrangement. Further models attempted to describe the structure in further dimensions. The Smith model described 5 collagen molecules arranged in a cylindrical manner, creating a concentric hollow structure known as a pentafibril. In this model the pentafibril is 3-3.5 nm thick with a length dependent on number of collagen molecules arranged longitudinally (Smith 1968). This model however required modification to allow for the larger scaled fibrils observed with SEM and AFM.
Hulmes and Miller proposed an alternative packing arrangement in which 5 collagen molecules pack in a pseudo-hexagonal structure (Hulmes and Miller 1979). A further development came in 1981 when Piez and Trus developed the previous models into a compressed pentafibril model. In this case 5 collagen molecules are again packed in a pseudo-hexagonal structure, however they are compressed in a tightly coiled left handed structure (Piez and Trus 1981).

More recent studies measuring electron density using X-ray diffraction have been used to construct the unit lattice for collagen (Orgel, Irving et al. 2006). It was found that although, like the Hodge and Petruska model, there are gap and overlap regions, the molecules are orientated in a 3 dimensional twisted manner as well as producing a staggered orientation. There have also been models suggesting collagen molecules arrange to form fibrils in a rope like structure, giving rise to the characteristic D-banding pattern irrespective of fibril diameter (Bozec, van der Heijden et al. 2007). Currently the conventionally accepted model is that presented by Orgel (Orgel, Irving et al. 2006) consisting of 5 collagen molecules twisted in a quasi-hexagonal lattice.

1.4 Collagenous tissue

1.4.1 Tendon

Tendons form a connection between muscle and bone, allowing the transfer of force. They are composed mostly of type I collagen surrounded by a proteoglycan and water extracellular matrix. Due to their structural properties, they are able to withstand loading, allowing force produced by the muscle to be transmitted with minimum dissipation as the tendon undergoes tensile stress. Some tendons also act as energy storage mediums, able to supply elastic energy much like a spring (e.g. the Achilles tendon) (Sawicki, Lewis et al. 2009).
The characteristics of the tendon can vary greatly depending upon the location and function of the tendon and its role in the surrounding muscular system. If the tendon is accompanied by large force producing muscles, the tendon is short and has a large diameter. Tendon properties include the ability to withstand compressive forces as well as tensile force. Tendon primarily rely on its tensile properties, and can often run along the side of a bone. The proximal tendon can also be called the muscle origin tendon and is a tendon connects the muscle to a fixed bone. The distal tendon can also be called the muscle insertion tendon and connects the muscle to the bone in motion.

1.4.2 Hierarchical structure

Collagen in tendon forms into structures and substructures at multiple length scales as shown in Figure 1.3. Tropocollagen (individual molecules of collagen) aggregate to form collagen molecules. These assemble (in the quasi staggered arrangement) to form subfibrils with diameters of 10 nm. These then fuse to form fibrils with diameters in the range of 10-350 nm (Graham, Holmes et al. 2000). A number of fibrils assemble to form collagen fibres with diameters up to 20 um and which can be viewed using light microscope. Collagen fibres are bound together to form primary fibre bundles or subfascicles. A number of subfascicles produce secondary fibre bundles or fascicles with a thickness of 50-300 um. It is the fascicles that produce the tertiary bundles which then bundle to form the tendon. It can be observed that there are multiple levels of substructure in the tendon and it is important to note that the cross sectional area of the various structures depends on the location and size of the tendon itself. The orientation of collagen fibres is dependent on the direction of force applied to the tendon. Due to the significant longitudinal application of force along the length of the tendon, the fibres are
generally orientated longitudinally. It has been shown that fibres can be oriented transversely to each other, forming overlaps (Józsa and Kannus 1997).

Surrounding the subfascicle, fascicle and tertiary fibre bundle are layers of endotenon, encasing the fibres. The endotenon is a fibrous sheath made up of thin fibrils of relatively uniform diameter. The endotenon contains vasculature, lymphatics and nerves allowing hydration of proteoglycans in the fascicles and also allows the opportunity for tendon fascicles to move along neighbouring fascicles without damage to the collagen fibres.

Figure 1.3: Ultrastructure of tendon (Józsa and Kannus 1997)

1.4.3 Ageing of tendon

There are multiple processes that occur to the various components of tendon as it ages. The absolute content of collagen remains the same however, due to the reduction in the content of other components within the extracellular matrix, the relative concentration of collagen increases (Ippolito, Natali et al. 1980, Kannus and Jozsa 1991, Tuite, Renström et al. 1997, Gosline, Lillie et al. 2002). Tendon collagen is also long lived, with a reduction
in an already low turnover resulting in increased retention of collagen molecules. This has the overall impact of age related slowing down of tissue repair.

Significant changes also occur to other components of tendon tissue including cells (tenoblasts) (Józsa and Kannus 1997), proteoglycans (decorin, aggrecan, versican), glycosaminoglycans (chondroitin sulphate, keratin sulphate, heparin sulphate) (Ryan, Sorushanova et al. 2015) and elastin (Gosline, Lillie et al. 2002).

The characteristics of cells are altered with age as tenoblasts convert into tenocytes (Józsa and Kannus 1997). The number of tendon cells decreases and changes to their shape occurs with cells becoming elongated (Ippolito, Natali et al. 1980, Nakagawa, Majima et al. 1994). The metabolic activity of tendon cells decrease thus reducing their protein synthesis rate (explaining the mentioned changes to collagen turnover) (Hayflick 1980, Ippolito, Natali et al. 1980).

Proteoglycan content decreases over time, causing a reduction in the water content of tissue. The concentration of extracellular water declines considerably, with changes to mechanical properties and the ability of tendons to glide being attributed to this (Ippolito, Natali et al. 1980, Tuite, Renström et al. 1997).

Elastin, a component of connective tissue that influences its mechanical properties, providing elasticity is significantly affected by age in tendon (Ippolito, Natali et al. 1980, Kannus and Jozsa 1991). The total elastin content of tissue reduces and elastic fibres have been observed to be enzymatically degraded (Robert, Moczar et al. 1974).
Human skin is primarily a protective organ, serving as the first defence against the external environment. The stratum corneum, the outermost layer of the epidermis, provides a physical barrier to the environment and mediates the permeability barrier function of the epidermis. Complementary to providing mechanical protection, UV-protection is provided by UV absorbing molecules such as melanin (Brenner and Hearing 2008) and vitamin D (Lehmann, Querings et al. 2004) and thermal protection provided by heat shock proteins being expressed by epidermal cells (Ghoreishi 2000). The epidermis also provides antimicrobial defence through expression of antimicrobial peptides, enzymes and chemokine by keratinocytes (Gunathilake 2015). Skin is also a sensory organ, allowing sensory inputs of force and temperature (Lumpkin and Caterina 2007). Finally, its other key role is to aid thermal regulation (Charkoudian 2003, Romanovsky 2014).

The skin dermal component is composed mostly of type-I collagen, secreted by dermal fibroblasts. The dermis is 2-3 millimetres thick, making up most of the thickness of our skin (80% of which is composed of type-I collagen) (Lovell, Smolenski et al. 1987). It is composed of two layers, the papillary dermis (a thin network mesh of type-III collagen) and reticular dermis (thick bundles of collagen type-I fibres). These undergo different age related changes (Terman and Brunk 2004). Other major components of the dermal extracellular matrix (ECM) include elastic fibres (elastin); which provide mechanical elasticity allowing responsiveness to physical loading (Lovell, Smolenski et al. 1987, Jeanmaire, Danoux et al. 2001), proteoglycans; decorin and versican, with a protein core and sulphated glycosaminoglycan (GAG) chains which are highly hydrophilic and provide water retention properties to the ECM (Tuite, Renström et al. 1997), and hyaluronic acid (HLA); a non-sulphated GAG chain which due to its hydrophilicity also has a great
capacity to bind with water providing hydration and water transport to the dermis (Ippolito, Natali et al. 1980, Reiser, Hennessy et al. 1987).

1.4.5 Ageing of dermis

Age related changes have a deleterious impact on the structure and functions of the skin (protection, regulation and sensation). Dermal ageing is a complex process with a number of changes occurring to non-collagenous components of the dermal ECM. Elastin content declines as it is produced less (Jeanmaire, Danoux et al. 2001), calcification of elastin fibres occurs, causing a deterioration in functional properties (Jeanmaire, Danoux et al. 2001). With elastin turnover approaching a lifespan, structural and physiochemical changes to the elastin network accumulate with age (Shapiro, Endicott et al. 1991, Langton, Griffiths et al. 2013). Decorin and versican undergo changes in their GAG chains, with studies showing a reduction in the molecular size of their polysaccharide chains (Tuite, Renström et al. 1997, Ryan, Sorushanova et al. 2015). HLA content in the dermis decreases as it is produced less by fibroblasts and disappears altogether in the epidermis as a function of age (Kobayashi, Ishii et al. 1994). HLA synthesised in the dermis also sees a decrease in the size of the GAG chain (Lovell, Smolenski et al. 1987).

Age related changes that occur to collagen at the molecular scale are elaborated on in the following sections.
1.5 Cross-linking

1.5.1 Hydrogen bonding

Polar groups found on the collagen molecule provide binding sites for hydrogen bonding. These hydration binding sites can result in the bonding of water molecules to the collagen molecule as well as hydrogen bonds between residues. Such hydrogen bonds can occur along the alpha chain (α and γ bridges), across neighbouring alpha chains (β and δ bridges) and across neighbouring molecules (ω bridges) (Bella, Brodsky et al. 1995).

1.5.1.1 Hydrogen bonding: Carbonyl groups

The triple helix of the collagen molecule is kept stable due to hydrogen bonding occurring between pro-alpha chains as shown in Figure 1.4 (left). A major source of these hydrogen bond is due to the polarity of carbonyl groups found on glycine, proline and hydroxyproline. Bonds can formed between N-H (amine group in a glycine) and C=O (carbonyl group in a proline), or between two carbonyl groups of residues in a neighbouring pro-alpha chain (Bella, Brodsky et al. 1995). This arises due to the inherent polarity of these groups and produces hydrogen bonding (figure 1.4, right).

Figure 1.4: Hydrogen bonding between different pro-alpha chains. Bonds form between adjacent pro-alpha chains (left) and non-adjacent pro-alpha chains (right), stabilising the triple helix (Shoulders and Raines 2009).
Molecular dynamics (MD) simulations have found that carbonyl groups from glycine provide a single hydration binding site while carbonyl groups from hydroxyproline provide double hydration sites. Proline carbonyl groups however, exclusively form an inter-chain hydrogen bond with amine groups (Bella, Brodsky et al. 1995).

The presence of glycine is essential in formation of the triple helix. Not only is this due to the size of the residue, but also due to the hydrogen bonding provided by glycine. In fact, there are a number of collagen disorders which occur due to mutations in the collagen gene disrupting the coding for glycine and resulting in a collagen triple helix in which glycine is replaced by other residues as in Osteogenesis Imperfecta (Beck, Chan et al. 2000, Bodian, Madhan et al. 2008).

1.5.1.2 Hydrogen bonding: Hydroxyl groups

Hydroxylation of proline also stabilises the triple helix by allowing the formation of another set of hydrogen bonds. Hydroxylation of proline (shown in Figure 1.5) requires the presence of prolyl hydroxylase to catalyse the reaction. The presence of hydroxyl groups provides a further set of hydrogen bond sites, with the ability to form triple binding sites being reported by MD simulations (Bella, Brodsky et al. 1995).

![Figure 1.5: Hydroxylation of proline in a proline-proline-glycine sequence.](image)
This in effect improves thermal stability of the molecule, and it has been reported that the addition of hydroxyproline results in a significant increase in the denaturation temperature of the collagen triple helix (Berg and Prockop 1973).

1.5.1.3 Water bridges

Hydrogen bonds formed can be directly between alpha chains within a collagen molecule, as well as between neighbouring collagen molecules. They can also be mediated by surrounding water molecules. In this case, “water bridges” between molecules ($\omega$) are formed in which there is a hydrogen bond from one molecule to a water molecule and subsequently from that water molecule to a residue on a collagen molecule thus binding the two collagen molecules together (Ravikumar and Hwang 2008). Water bridges can have a number of water molecules mediating the hydrogen bond, with the anchoring residues dictating the number of water molecules that form along the chain (Bella, Brodsky et al. 1995).

Hydrogen bonds have been shown by molecular dynamics simulations as having two roles; one sub-molecular, forming intra-chain bonds and stabilising the triple helical structure. Another role in the mesoscale, forming intermolecular bonds, mediating self-assembly of molecules into larger structures (Ravikumar and Hwang 2008). Hydrogen bonding plays a crucial role in collagen fibrillogenesis (Leikin, Rau et al. 1995).
1.5.2 Covalent cross-links

1.5.2.1 Lysyl Oxidase mediated

In addition to hydrogen bonding, covalent cross-links also form between residues and conserve the triple helical assembly. Once propeptides of the tropocollagen molecule have been cleaved into short non helical telopeptides and fibrillogenesis occurs, lysine residues at the N and C termini of the tropocollagen molecules are exposed and form bonds with hydroxylysine residues under the presence of lysyl oxidase. Lysyl oxidase (LOX) is an enzyme found in the ECM, secreted by fibroblasts in the dermis and keratinocytes in the epidermis. Following cleavage of collagen N-telopeptide and self-assembly of collagen molecules into fibrils, lysine and hydroxylysine residues undergo conversion into reactive aldehydes which react with other aldehydes or amine groups to form immature cross-links, eventually involving further amino acids to form mature covalent cross-links such as Pyridinoline (Saito and Marumo 2010). These cross-links form as stabilisers to both the triple helical complex as well as supramolecular fibrils formed by these molecules.

1.5.2.2 Advanced Glycation End product cross-links

As collagen is secreted into the extracellular matrix of various tissues where it forms supramolecular structures, it is exposed to glucose and can undergo modification. In the case of glycation, these modifications are the non-enzymatic reaction of the aldehyde group from glucose (or other sugars) with amine groups found on the collagen side chains. If exposure to glucose is chronic, further oxidation occurs and advanced glycation end products (AGE’s) can form. Some of these AGE’s result in a form of covalent cross-links forming between residues as they are linked by the AGE’s (Avery and Bailey 2006).
Glucosepane is the most common AGE cross-link (covalently cross-linking Lysine and Arginine). It is formed due to glucose, the highest concentration sugar found in vivo (Monnier, Sun et al. 2014).

Glucose exists mostly in its less reactive closed chain form, in contrast, its more reactive open chain form only makes up 0.002% of glucose molecules in vivo, requiring a prolonged exposure to glucose for a significant number of molecules to become cross-linked (Okano, Masaki et al. 2002).

The reaction begins with the Mailliard reaction (Dyer, Dunn et al. 1993, Monnier, Mustata et al. 2005, Avery and Bailey 2006). In this case, the aldehyde group from glucose reacts (reversibly) with the amine group of lysine (Figure 1.6a). A Schiff base is formed which is unstable and must undergo further rearrangement. The Schiff base contains a double bond between a carbon (from the glucose) and nitrogen (from lysine) with an alkyl group attached to the nitrogen (Figure 1.6b). The Schiff base then undergoes an Amadori rearrangement where the double bond moves along the chain until a carbonyl group forms, resulting in the formation of a ketone. This new complex is called an Amadori product (Figure 1.6c).
The Amadori product then over the course of a number of weeks undergoes a number of intermediary steps which have not yet been fully determined (Sjöberg and Bulterijs 2009, Monnier, Sun et al. 2014). Eventually, the open chain product forms a closed ring with a nitrogen hydrolysing with an Arginine residue side chain, forming Glucosepane (Figure 1.7).
There are a number of other AGE cross-links that can also form due to the non-enzymatic glycation of collagen shown in figure 1.8. Pentosidine also cross-links Lysine and Arginine (Aoki, Yazaki et al. 1993), formed due to the ribose (a simple sugar) and is a common AGE often used as a biomarker for diagnosing AGE accumulation due to its fluorescence (Nomoto, Yagi et al. 2013). Other cross-links include DOGDIC (deoxyglucosone-derived imidazolium cross-link - cross-linking Arginine-Lysine), MOLD (methylglyoxal derived lysine dimer) and GOLD (glyoxal derived lysine dimer), both are bis(Lysyl) cross-links between Lysine-Lysine (Liu, Zhao et al. 2004, Alikhani, Alikhani et al. 2005). These are formed from deoxyglucosone, methylglyoxal and glyoxal respectively. Deoxyglucosone, methylglyoxal and glyoxal are formed due to fragmentation of the Amadori product (and thus are degradation products of the Malliard reaction) and are highly reactive. They are however found in minute concentrations in vivo and so these AGE cross-links are not as abundant as Glucosepane (Avery and Bailey 2005). The most common glyoxal and methylglyoxal based AGEs are hydroimidazonones (G-H1 and MG-H1, respectively) - these adducts are not cross-links however and instead bind to arginine (Ahmed, Argirov et al. 2002, Thornalley, Battah et al. 2003).
1.6 AGEs and ageing of collagen

AGE cross-links and other products are a major contributing factor to one of the ageing pathways discussed at the beginning of this chapter – alteration of protein due to accumulation of waste. Upon secretion of collagen into the ECM for assembly into supramolecular structures, collagen can undergo the irreversible non-enzymatic Maillard reaction as it is exposed to glucose or other reducing sugars. This leads to the accumulation of a multitude of AGEs with age (Robert, Moczar et al. 1974), forming not only products but also cross-links between collagen molecules. As mentioned previously, glucosepane is the most common AGE cross-link as the highest concentration sugar found in vivo is glucose (Monnier, Sun et al. 2014) and so the major age related alteration that occurs to collagenous tissue is the accumulation of glucosepane.
1.6.1 Other age related changes to collagen

Both collagen and elastin undergo enzymatic cross-linking in the ECM as a function of ageing (Monnier, Mustata et al. 2005, Saito and Marumo 2010). LOX activity has been shown to be higher in human skin than in other tissues (Kuivaniemi 1985) with an increase in expression in aged human skin (Langton, Griffiths et al. 2013). However, the proportion of LOX derived cross-links compared to other covalent cross-links in extracellular tissue reduces with age (Szauter, Cao et al. 2005). The proportion of non-enzymatic (glycation) cross-links investigated in this study increases and thus is associated with ageing rather than the enzymatic LOX derived cross-links. LOX not only maintains collagen alignment and fibril structure (Bailey 2001), but is also crucial for maintaining homeostasis of elastin, with cross-linking preventing excessive elasticity of fibres as well as aiding spatial deposition of elastin (Liu, Zhao et al. 2004).

1.6.2 Effect of AGEs on components of dermal ECM

AGE accumulation affects other components of dermal ECM (Figure 1.9), including dermal and epidermal homeostasis (Pageon, Zucchi et al. 2015). Firstly, cell activity can be drastically altered as glycation products bind to the Receptor for Advanced Glycation End products (RAGE) triggering fibroblast and keratinocyte apoptosis through binding of CML (Carboxy-Methyl-Lysine, an adduct) to RAGE (Alikhani, Alikhani et al. 2005) and senescence (Ravelojaona, Robert et al. 2009). Cell signalling is also affected with the release of pro-inflammatory and pro-fibrotic cytokines (Uchiki, Weikel et al. 2012). Secondly, AGE binding to cells can lead to production of reactive oxygen species (ROS), which can lead to acceleration of further AGE production (Wondrak, Roberts et al. 2002, Wondrak 2007). When coupled with exposure to UV radiation (extrinsic ageing, brought
upon by environmental conditions), AGEs such as pentosidine can act as photosensitizers, accelerating oxidative damage, resulting in increased damage in older skin (Bastien P 2013). Thirdly, synthesis of ECM components are also affected by RAGE binding, with gene expression of MMPs and integrin being upregulated (Molinari, Ruszova et al. 2008, Pageon, Zucchi et al. 2015), fibronectin downregulated (Pageon, Zucchi et al. 2015). Synthesis of various collagen chains are affected (Pageon, Zucchi et al. 2015) and glycation of collagen can have a number of effects on its functional properties, increasing its resistance to digestion by enzymes (DeGroot, Verzijl et al. 2001, Wondrak 2007).

Figure 1.9: Age related changes to components found within dermal/epidermal ECM due to intrinsic and extrinsic ageing processes.
1.6.3 Glucosepane and collagen turnover

Endogenous glucosepane accumulation happens gradually over time as the extracellular matrix of various tissues become cross-linked due to long term exposure to glucose (Sell, Biemel et al. 2005). This is however a slow rate process and a number of other factors affect the level of glucosepane cross-linking in tissue. Glucose exists mostly in its less reactive closed chain form. In contrast, its more reactive open chain form only makes up 0.002% of glucose molecules in vivo, requiring a prolonged exposure to glucose for a significant number of molecules to become cross-linked (Sjöberg and Bulterijs 2009).

This requirement leads on to another factor determining accumulation. Collagen molecules are secreted by cells and digested by enzymes at different rates in various different tissues. This turnover rate affects the level of cross-linking that will form over time. If the turnover rate is high, new collagen is secreted at a faster rate and significant amounts of collagen will not become cross-linked, reducing glucosepane accumulation (Verzijl, DeGroot et al. 2000). This leads to different concentrations of glucosepane (and other AGEs) being detected in different collagenous tissues (Table 1.2).
The formation of AGEs are also highly associated with the condition of diabetes due to the sustained high concentrations of glucose in the bloodstream of those suffering from the condition. An inability of the body to control the blood glucose concentrations often leads to hyperglycaemia, significantly increasing the likelihood of Glucosepane production (as well as other AGEs). This is reflected in the increased concentrations of Glucosepane measured in various tissues shown in Table 2.

The rate of collagen turnover as well as the concentration of blood glucose are both factors affecting the degree of accumulation (Szauter, Cao et al. 2005).
1.7 Research question
Ageing of tissue comes in many forms, impacting on many components, often in an interdependent system. It is important to break these processes down to understand their individual effects. The most significant and obvious biochemical change to the most important extracellular protein is glycation of collagen. This study aims to consider the fundamental impacts of glycation on collagenous tissue, by studying the nanoscale mechanobiology of ageing. This will require an in-vitro approach to isolate the impact of glycation on collagen amongst other ageing processes. It will then require an ex-vivo approach to study the occurrence of markers of ageing identified by the in-vitro study.

1.8 Objectives
Understanding the nanoscale mechanobiology of ageing of collagenous tissue involves investigating collagen at its molecular and quaternary structure. This requires the development of a variety of novel techniques such as;

- Identify methodologies to characterise the nanoscale impact of AGE accumulation on the collagen matrix.
- Develop an in-vitro glycated tissue model, isolating the various processes of ageing due to the accumulation of AGE cross-links on the collagen matrix.
- Apply the methodologies developed to compare and contrast the in-vitro model to ex-vivo tissue samples of dermis and tendon.
2 Analytical techniques

2.1 Identifying characterisation techniques

There are two primary routes of investigation to determine structural and biophysical properties of collagen; morphology and mechanics at the nanoscale. As discussed in Chapter 1, collagen takes a fibrillar form at the nanoscale and there are few techniques appropriate for such characterisation. A comparison of macroscale and nanoscale techniques is provided below.

2.2 Investigating morphology

2.1.1 Optical microscopy

Optical techniques such as light microscopy and fluorescence microscopy (Ber, Torun Köse et al. 2005, Kenar, Köse et al. 2006) are commonly used to characterise in-vitro tissue engineering models however the resolution is limited by the diffraction limit of light to resolve features at the macroscale (200nm) meaning individual collagen fibrils cannot be resolved. Light microscopy typically involves histology – requiring thin slices of the in-vitro tissue model and mounting onto a microscope slide. Stains can be utilised to identify components of interest within the matrix (Alturkistani, Tashkandi et al. 2016). Extended optical microscopy techniques such as confocal microscopy (Burdick and Anseth 2002, Unger, Wolf et al. 2004, Svensson, Nicklasson et al. 2005) and two-photon microscopy (Schade, Weiss et al. 2010, Villa, Wang et al. 2013) augment spatial resolution, while this is sufficient for imaging of bulk tissue matrix and cells, this study requires higher resolution imaging.
2.1.2 Tomography

Tomography techniques such as Optical Coherence Tomography (OCT), Magnetic Resonance Imaging (MRI) and Micro Computerised Tomography (µCT) have been used to visualise tissue engineered constructs in three dimensions. Limits in spatial resolution due to the diffraction limit of light apply to OCT and so it is typically used to investigate cell proliferation through a tissue engineered scaffold (Ying, Arnaud et al. 2006, Zheng, Rupnick et al. 2009) rather than ultrastructure of a scaffold matrix. MRI has been used extensively to investigate in-vitro scaffolds (Hickethier, Kroger et al. 2016). Providing high contrast for soft tissues, MRI can produce 3D imaging at a resolution of 5 µm, which although excellent, is still insufficient to visualise collagen structure at the nanoscale. The use of µCT is similar in that spatial resolution is insufficient, however the technique also requires the use of often contrast agents to image soft tissues, which can have toxic effects in-vivo (Faraj, Cuijpers et al. 2009).

2.1.3 Electron microscopy

Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) are commonly used characterisation techniques that provide high resolution visualisation of tissue engineered constructs. SEM is able to resolve features 10 nm in size with TEM providing 0.2 nm resolution. SEM is used widely to visualise the microstructure of tissue engineered scaffolds, cells and a variety of other biological samples (Cuijpers, Walboomers et al. 2011). With the ability to resolve individual collagen fibrils, SEM presents a strong candidate for identifying morphological markers of ageing of collagen at the nanoscale (Hulmes, Jesior et al. 1981, Provenzano and Vanderby 2006, Alexander, Daulton et al. 2012). Using scanning and transmission electron microscopy it is possible
to observe nanoscale features including D-banding periodicity of a collagen fibril (Li and Aparicio 2013, Starborg, Kalson et al. 2013).

A crucial consideration factor however, is sample preparation and sample environment. Tissue models require dehydration, fixing (and resin embedding for transmission electron microscopy, TEM) as well as coating of the surface with a conductive layer to be imaged (SEM). For both SEM and TEM, the sample must also be placed in vacuum during electron beam irradiation. The sample therefore undergoes highly invasive processes before imaging which means neither in-vitro or ex-vivo samples are in physiologically relevant conditions. The destructive, irreversible nature of sample preparation also renders samples unusable for further tissue culture or analysis using other techniques.

2.1.4 Atomic force microscopy

In contrast to electron microscopy, AFM is a non-destructive technique which gained fast recognition for its ability to obtain images with nanoscale resolution whilst requiring little to no sample preparation (Chernoff 1992, Baselt, Revel et al. 1993, Thalhammer, Heckl et al. 2001). Tissue can be dissected to obtain sections, which once dehydrated in air can be imaged without a vacuum in standard room temperature and pressure. As long as the surface roughness of the sample is in the order of microns, it is possible to obtain high resolution imaging resolving collagen ultrastructure. Fibrils and features such as D-banding periodicity from rat tail tendon have been imaged by a number of studies (as shown in Figure. 2.1) by gently pulling out fibres from the tendon and placing on a glass substrate (Baselt, Revel et al. 1993, Bozec, van der Heijden et al. 2007, Bozec and Odlyha 2011). Indeed subfibrillar structure have also been observed in regions with locally unwound fibrils (similar to rope unwinding) allowing further models for the ultrastructure of collagen fibrils to be proposed (Bozec, van der Heijden et al. 2007). AFM

Further to this, imaging can also be done in liquid, providing close to physiological conditions (when compared to the vacuum required for electron microscopy) (Raspanti, Congiu et al. 2001). Developments in video rate AFM allow the possibility of observing cellular interactions, giving AFM a unique advantage over conventional imaging techniques (Picco, Bozec et al. 2007).

In order to achieve nanoscale characterisation of the morphology of collagen fibrils, atomic force microscopy was employed as the primary technique in this study. Air-dried tissue models were prepared and imaged in contact mode as described in the methods section. It was important to ensure that any potential heterogeneity in the glycated tissue models would not bias the imaging and so each hydrogel was imaged at multiple locations spanning four different regions (the hydrogels were split into 4 quadrants) to obtain a representative characterisation of nanoscale morphology.
2.3 Investigating nanomechanical properties

There are a number of techniques that are used to investigate mechanical properties of in-vitro tissue models. These include uniaxial loading (Duprey, Khanafer et al. 2010), biaxial loading (Bilgen, Chu et al. 2013), micro-indentation and tensile testing (Griffin, Premakumar et al. 2016) using customised rigs or commercially available, standard testers such as Instron. While these techniques provide an understanding of macroscale properties of tissue and in-vitro models, the requirement for nanoscale understand in this study leads us to consider the use of nanoindentation using AFM.

2.1.5 AFM Nanoindentation

A feature of AFM is the ability to interact directly with the sample. Because the AFM tip can be of nanoscale size, it can be used to apply stress to remarkably small features in the sample (such as individual fibrils) to study mechanical properties such as the elastic modulus, E. There are 3 major types of nanoscale mechanical testing of collagen using AFM; i) nanoindentation of fibrils, ii) extension of individual fibrils and iii) extraction of individual collagen molecules from fibrils.

2.1.6 Nanoindentation in literature

A number of studies (listed in Table 2.1) have applied the nanoscale radial indentation to collagen fibrils to measure their stiffness transverse to fibril plane. This involves the tip causing a physical indentation in the surface of the collagen fibril as shown in Figure. 2.2. Measuring the force required to produce a certain amount of indentation gives an indication of the stiffness of the fibril.
Other modes of nanomechanics include 3-point bending and direct fibril extension (described in the appendix section). The relative ease of the nanoindentation method makes it a more suitable technique to use for investigation of nanoscale mechanical properties. It does not require a specialised substrate (as in the case of the 3-point bending technique) and does not require modification of the nanoscale feature being investigated (as in the case of direct fibril extension). An important point to take into consideration for fibril nanoindentation is the fact that fibrils are indented radially - therefore nanoindentation measures the transverse stiffness of a collagen fibril.

Figure 2.2: AFM tip radially indenting a collagen
<table>
<thead>
<tr>
<th>Authors</th>
<th>Sample</th>
<th>E (GPa) Dry</th>
<th>E (MPa) Wet</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Wenger et al., 2007) (Wenger, Bozec et al. 2007)</td>
<td>Rat tail tendon (Type I collagen)</td>
<td>3.75 - 11.5</td>
<td>-</td>
</tr>
<tr>
<td>(Tang et al., 2014) (Tang, Fong et al. 2014)</td>
<td>Cartilage (Type II collagen)</td>
<td>1.03 - 3.23</td>
<td>8.59 - 11.24</td>
</tr>
<tr>
<td>(Heim et al., 2006) (Heim, Matthews et al. 2006)</td>
<td>Sea cucumber (Type I collagen)</td>
<td>1 - 2</td>
<td>-</td>
</tr>
<tr>
<td>(Yadavalli et al., 2010) (Yadavalli, Svintradze et al. 2010)</td>
<td>In vitro type I collagen (extracted from calf skin)</td>
<td>1.03 ± 0.31</td>
<td>-</td>
</tr>
<tr>
<td>(Grant et al., 2008) (Grant, Brockwell et al. 2008)</td>
<td>In vitro type I collagen (extracted from bovine achilles tendon)</td>
<td>1.9 ± 0.5</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>(Grant et al., 2012) (Grant, Twigg et al. 2012)</td>
<td>Skin (Type I collagen)</td>
<td>-</td>
<td>0.025 - 5.7</td>
</tr>
</tbody>
</table>

Table 2.1: Studies measuring radial stiffness of fibrils using nanoindentation
2.1.7 Modelling collagen nanoindentation

There are two commonly used models used for analysis of force displacement curves for calculating $E$; the Hertzian model and the Oliver-Pharr adapted, Sneddon model.

2.1.8 Hertzian model

The Hertzian model was developed to describe contact forces between two elastic spheres with different radii. The model was then extended to describe contact of a sphere with an elastic half space (a surface with an infinite thickness) and relies on a number of assumptions;

- The sample surface must be solid, frictionless and smooth
- Sample is isotropic and homogenous (constant $E$)
- Sample is an elastic solid with no plastic deformation
- Indentation depth is negligible compared to sample thickness (infinite half space)
- The indenter is non deformable
- There are no other interactions between tip and sample (i.e. no adhesion or repulsive forces)
- There is no occurrence of creep

The following equation (Lin, Dimitriadis et al. 2007) is used for calculating $E$;

$$E = \frac{P \pi (1-v^2)}{\delta \ 2\delta ta} \quad (1)$$

Where;
P: Load applied

δ: Tip distance (taken as the difference in height measured from the contact point to the point at which the maximum force load is reached)

v: Poisson’s ratio (0-0.5 for a solid, taken as 0.5 for biological samples)

α: Semi opening angle of tip

The variables to be obtained from the F-D curve are shown in Figure 2.3;

![Figure 2.3: Variables required from the F-D curve for Hertzian modelling](image)

2.1.8.1 Limitations of Hertzian modelling

Although the Hertzian model is the conventional model used in literature (Heim, Matthews et al. 2006, Yadavalli, Svintradze et al. 2010, Tang, Fong et al. 2014), there are a number of limitations involved with using this model. These relate to the assumptions made;

- The sample surface is not frictionless and smooth
• Sample is not an elastic solid and will undergo a small amount of plastic deformation
• Indentation depth is negligible compared to sample thickness (infinite half space)
• The cantilever itself will deflect as the probe indents the sample
• There are also adhesive and repulsive interactions between tip and sample
• There is no compensating factor that takes into account effective contact area
• The model does not take into account sinking in of wider surface during indentation (rather than a localised indentation at the tip).
• Biological samples often have inhomogeneous elasticity (cell membranes etc). In the case of collagenous tissue this may relate to inhomogeneity in the fibrillar network.

2.1.9 Oliver-Pharr adapted Sneddon model

The Oliver-Pharr model was adapted from the Sneddon model primarily for describing the mechanics of thin films (in contrast to the Hertzian which requires an infinite half space) (Oliver and Pharr 1992). The Oliver-Pharr model takes into account the deformation of the sample surface as a load is applied and models this by introducing the concept of an effective contact area (changing as function of indentation depth –Figure. 2.4) and a “sink in” factor (sinking in of the entire surface surrounding the indentation point).
As the tip begins to indent the surface, the contact area of the tip that is exposed to the surface will change as a function of indentation depth. The overall process can be described by Figure. 2.5;

\[
E = \sqrt{\frac{p}{2\sqrt{A(h_0)}}} \quad (2)
\]

Where;

\[
h = h_{\text{max}} \text{ (maximum indentation depth)} \quad (3)
\]

\[
h_z = \varepsilon \frac{P_{\text{max}}}{S} \quad (4)
\]
The sink in depth - the depth the entire surface that will sink in when loaded

\[ h_c = h_{max} - h_s \quad (5) \]

\[ h_c = h_{max} - \varepsilon \frac{P_{max}}{S} \quad (6) \]

\[ \beta = 1.2304(1 - 0.21v - 0.01v^2 - 0.41v^3) \quad (7) \]

Where \( \varepsilon \) is determined by the shape of the indentor and is given by;

\[ \varepsilon = m \left[ 1 - \frac{2\tau}{\sqrt{\pi}} \left( \frac{m}{2(m-1)} \right) (m-1) \right] \quad (8) \]

\( \varepsilon \) represents the shape of the indentor (the tip geometry) and is accepted in literature as 0.75 at small indentation depths (Oliver and Pharr 1992).

The effective contact area can be calculated with the following equation (which depends on the tip shape) (Oliver and Pharr 1992);

\[ A = \sum_{n=0}^{8} C_n (h_c)^{2-n} = C_n h^2 + C_1 h + C_2 h^{1/2} + C_3 h^{1/4} + \ldots C_8 h^{1/128} \quad (9) \]

Where \( C_i \) is a constant which depends on the shape of the indenter.

2.1.10 Tip-Sample separation correction

The indentation depth is measured by the deflection of the laser spot on the photodiode. However there is also another factor which also causes deflection of the laser; cantilever deflection. In fact, as a force is applied to the sample, the cantilever itself will begin to bend. This bending also has a contributing factor to the overall deflection of the laser.

Therefore, the indentation height that is output in the F-D curve can be described as;
\[ h_F = \delta_F + x_F \quad (10) \]

Where \( h \) is the height at any given force, \( \delta_F \) is the indentation depth into the sample at any given force and \( x_F \) is the cantilever deflection at any given force. Therefore;

\[ \delta_F = h_F - x_F \quad (11) \]

The cantilever deflection can be calculated from knowing its spring constant and applying Hook's Law;

\[ F = kx_F \quad (12) \]

\[ x_F = F / k \quad (13) \]

And so \( x_F \) can be calculated at every point of the F-D curve and subtracted from \( h_F \) to give the actual penetration depth at any given force \( \delta_F \).

This results in a narrowing of the F-D curve as the unnecessary component of the height measured is removed (Figure. 2.6).

![Figure. 2.6: F-D curve after the tip-sample separation correction (dashed line)](image-url)
2.4 Investigating single molecule mechanics: Single molecule force spectroscopy (SMFS)

In addition to nanoindentation, it is important to probe into the mechanical properties of collagen at a more fundamental level. It is possible to investigate individual molecular interactions through the use of AFM single molecule force spectroscopy (SMFS). SMFS is a technique that has emerged as a powerful tool for direct manipulation of biological samples. AFM SMFS on collagen samples involves resting an AFM tip on a collagen fibril to allow adhesion of collagen molecules to the tip. The AFM tip is then retracted from the surface of the fibril, bringing weakly bound collagen molecules along with it. Due to the weak hydrogen bonding between the AFM tip and collagen molecule, after a certain distance collagen molecules will unbind from the tip due to their stronger affinity with neighbouring collagen molecules. This distance can be measured in the form of a contour length, giving the length at which the collagen molecule was pulled away from neighbouring molecules before detaching. It is important to note that this is not a stretching of the molecule, but a pulling due to the weak tip-collagen affinity. Single molecule pulling experiments can give a greater understanding of the effect of cross-linking by more directly measuring their effect on molecular binding properties.

2.1.11 SMFS in literature

There are a number of instruments other than AFM which can be used for SMFS such as optical and magnetic tweezers. Optical tweezers provide higher spatial resolution (0.1-2 nm) as well as a lower interaction force range (0.1-100 pN) than AFM (with a spatial range of 0.5-1 nm and interaction force of 10-10^4 pN). The limitations of the optical tweezers method however include the risk of damaging biological samples through
heating (Peterman, Gittes et al. 2003) and photo-damage (Neuman, Chadd et al. 1999). AFM SMFS has been used widely to investigate affinity and stretching properties of a variety of biomolecules such as polysaccharides (Marszalek, Li et al. 2002), and proteins such as titin (Rief, Gautel et al. 1997, Tskhovrebova, Trinick et al. 1997), DNA (Lee, Chrisey et al. 1994, Strunz, Oroszlan et al. 1999) and collagen molecules (Gutsmann, Fantner et al. 2004, Bozec and Horton 2005). In the mentioned cases the molecules were chemically bound to the tip, investigating tensile properties of molecule, unlike the aims of this study.

2.1.12 Contour length in literature

Contour length of a biopolymer chain can be calculated using a variety of models including the freely jointed chain (FJC) model and the worm-like-chain (WLC) model (also known as the Kratky-Porod model). Examples of the use of the FJC model includes the calculation of contour length of titin molecules after stretching with optical tweezers (Tskhovrebova, Trinick et al. 1997) and the measurement of DNA molecule elasticity using magnetic beads (Smith, Finzi et al. 1992). The WLC model has been applied to a variety of biopolymer molecules such as tenascin (Oberhauser, Marszalek et al. 1998), spectrin (Rief, Pascual et al. 1999), DNA (Marko and Siggia 1995, Seol, Li et al. 2007).

The WLC in particular has been used to calculate contour length of collagen using AFM SMFS. Bozec et al. (Bozec and Horton 2005) characterised single collagen molecules adsorbed on a mica substrate before undertaking single molecule pulling experiments to obtain contour length of collagen using both imaging and SMFS. A contour length of 287 ± 35 nm was obtained through imaging and 202 ± 5 nm through single molecule pulling. Single molecule pulling of native tendon was carried out by Gustmann et al. (Gutsmann,
Fantner et al. 2004), however in this instance a rupture length rather than contour length was measured. This described the distance in the force-distance curve between successive rupture events, which were described as bond breaking events. Peak rupture lengths of 78 nm and 22 nm were reported, indicating the presence of two types of periodicity. In comparison to this, Sun et al. found the contour length of type I collagen molecules using optical tweezers to be 309 nm (Sun, Luo et al. 2002).

2.1.13 Modelling collagen using the worm-like chain (WLC) model

The collagen molecule can be thought of as a chain of inflexible components (in this case residues) which is unfolded until it reaches its contour length – the maximum length it can be extended. The molecule can be modelled using two prevailing classical models; the freely jointed chain (FJC) model and the worm-like chain (WLC) model. The FJC model describes a collagen molecule as being a series of rigid subunits attached to each other via flexible hinges. The subunits are discrete residues and are assumed not to interact with each other, leading to a random orientation of subunits driven by thermal fluctuations. The molecule can be represented as a random walk with each subunit representing a step. The molecule reaches an equilibrium at maximum entropy with deviations from this arrangement (in the form of molecule unfolding) requiring energy. This describes molecule elasticity as being driven by entropy, an entropic elasticity.

The FJC model does not accurately portray a biopolymer molecule such as collagen as in reality, there is conformity between successive residues (subunits). The limited freedom of movement of neighbouring residues arises in a “persistent length” of a molecule. This describes the tangential length along a molecule that encompasses several residues while maintaining the same curvature (or gradient). The persistent length can be imagined as
a quasi-subunit of uniform curvature and for the collagen type I molecule was calculated to be 14.5 nm by Sun et al (Sun, Luo et al. 2002). The numerical interpolation relating applied force $F$, persistent length $q$ and contour length $L_0$ is given by;

$$F = \frac{kT}{q} \left( \frac{1}{4(1-\frac{x}{L_0})^\gamma} - \frac{1}{4} + \frac{x}{L_0} \right)$$ (14)

### 2.5 Summary

There has been a wealth of investigation carried out using these techniques in a variety of studies. This study will endeavour to apply these techniques in the context of ageing of collagenous tissue, bringing a new perspective to the field. This study will apply the techniques identified in both an in-vitro and ex-vivo setting.
3 Materials and methods

3.1 Introduction

There were a variety of tools and techniques used throughout the course of this research project. The methodologies are described in the following sections.

3.2 Plastically compressed collagen (PCC) models

Type-I collagen (2.1mg/ml collagen in 0.6% acetic acid, from FirstlinkUK) was used. 10x MEM (Minimum essential medium) was added as an indicator of pH to aid neutralisation (0.4ml MEM for 3.6ml collagen). The solution was neutralised using 5M NaOH and 1M NaOH. 5M Sodium Hydroxide added drop by drop until the solution went from translucent pale yellow to translucent pink in colour. 1M Sodium Hydroxide was then added drop by drop until the solution reached a translucent dark pink colour once neutral (taking care not to add excess Sodium Hydroxide).

Once neutralised the low viscosity gel was placed in a stainless steel casting mould (approximately 5mm thick) as shown in figure 3.1(a). This was incubated at 37°C for 30 minutes to allow settling to occur and cross links to form, increasing the viscosity of the gel. After incubation the gel was removed from the cast, to leave a viscous gel as shown by figure 3.1(b).

![Figure 3.1: Neutralised collagen hydrogel in a mould (a) and after incubation (b)](image)
The gel was placed onto a thin nylon mesh, ready to be plastically compressed. The gel was placed onto a filter paper, a stainless steel mesh, and nylon mesh, and covered with a second nylon mesh, as shown in figure 3.2(a). The mould was placed onto a glass slide and applied to the top of the gel for 5 minutes to allow compression to take place, removing water from the gel, illustrated by figure 3.2(b). Upon compression, the gel was then rinsed in water and placed in 20ml PBS (phosphate buffered saline) for storage, shown in figure 3.2(c).

Figure 3.2: The various stages involved in producing a plastic compressed collagen model setting a gel (a), compression (b) and storage (c)
Real Architecture for 3D Tissues (RAFT)

This RAFT method is similar to the PC method pre-incubation, with the primary difference being the neutralisation of collagen in the wells of a cell culture plate and removal of water using stabilisers. In this method, collagen solution was neutralised in a 75 cm cell culture flask as shown in figure 3.3(a). The neutralisation was carried out in cooled environment using an ice bath in order to slow down pH transition and prevent the addition of excess NaOH. The neutralised collagen solution was then transferred into a 12 well tissue culture plate as shown in figure 3.3(b) and allowed to incubate for a period of 30 minutes to allow setting. Post incubation, the RAFT method employs the use of stabilisers produced from filter paper to remove excess water from the hydrogel through capillary action, as shown in figure 3.3(c). The collagen scaffold is then ready for further processes to initiate glycation.

Figure 3.3: The various stages of producing RAFT tissue models including neutralisation (a), separation into well plates (b) and then stabilisation (c).
3.3 In-vitro glycation

Glycation of in-vitro collagen gels was carried out through the addition of three different agents; glucose, glyoxal and methylglyoxal. The glycating agent was added to 20 ml PBS solution in 50 ml falcon tubes and the tube mixed thoroughly to ensure full dilution. The collagen gel was then added to the solution and incubated at 37°C for the duration of the experiment. The collagen gel was shaken within the falcon tube every 2 days.

A glucose solution for glycation was produced by adding 28.6 g D-glucose to 220 ml PBS (maximum solubility concentration) for a 722 mM glucose solution. A methylglyoxal solution for glycation was produced by adding 2.14 ml methylglyoxal (1.17 g/ml) to 20 ml PBS to produce a 100 mM solution. A glyoxal solution for glycation was produced by adding 1.98 ml glyoxal (1.265 g/ml) to 20 ml PBS to produce an 800 mM solution.

3.4 Sample preparation of collagen gels

Collagen gel models which were submerged in PBS solution (with or without glycating agents) were rinsed in UHQ water (filtered using a 0.22 µm syringe filter) for several minutes using tweezers to allow full flow of water over all parts of the gel. The gels were then dissected into 5x5 mm squares, rinsed again in fresh, filtered UHQ water and mounted onto glass microscope slides which had been coated in poly-l-lysine and left to air dry over a period of 3 hours. The mounted gels were kept in a fume hood and allowed to air dry completely before storage in a refrigerator at 4°C.
3.5 **Ex-vivo tissue collection**

**Human skin**

Skin tissue was collected (REC ref 6398) by Dr Stratton (Royal Free Hospital) by taking punch biopsies from volunteers. The punch biopsies were taken from a location above the elbow from the side of the arm less exposed to the sun. The tissue was then snap frozen using the following method;

1. A container containing isopentane was placed in liquid nitrogen where it is super cooled.
2. The tissue biopsy was placed in an Eppendorf which is submerged in the isopentane.
3. The Eppendorf was then stored at -80°C until sample preparation.

**Human Achilles and Anterior tibialis tendon**

Tendon tissue was collected (REC ref. 09/H0304/78) by Professor Birch (Stanmore) and dissected into smaller sections before being snap frozen using the following method;

1. A container containing hexane was placed on dry ice for 30 minutes to allow the hexane for super cooling. Dry ice was then added to hexane.
2. Cryo-embedding medium (Tissue Tek) was added to the base of a cryo-mould.
3. The tendon section was paced onto the cryo-mould containing the medium.
4. The cryo-mould was then placed into the hexane using forceps. Once the Tissue Tek started to freeze the cryo-mould was dropped into the hexane until completely frozen.
5. The frozen section was wrapped in foil and stored at -80°C until sample preparation.
**Equine superficial digital flexor tendon**

Tendon tissue was collected by Prof. Birch (Stanmore) and dissected before being stored at -20°C until sample preparation.

**Rat tail tendon**

Rat tail tendon was collected by Dr Bozec (Eastman Dental Institute) and stored at -20°C until sample preparation.

### 3.6 Cryosectioning of ex-vivo tissue

Tissue was cryosectioned as using following protocol;

1. Samples were stored in dry ice whilst transporting to Blizard Core Pathology, Royal London Hospital for cryosectioning.
2. Preliminary dissection of the snap frozen tissue was required in order to allow mounting into the microtome. Tissue was partially thawed at room temperature in order to allow ease of dissection. Dissection was carried out using a surgical scalpel.
3. Cryo-embedding medium (Bright cryo-m-bed) was placed into a cryo-mould.
4. The dissected tissue was placed in the cryo-mould containing the medium and the cryo-mould was placed in -20°C to allow the medium to set as shown in figure 3.4.
5. After setting, the embedded tissue was then mounted in a cryostat (Thermo scientific) which had been cooled to -20°C. Cryo-spray (Cell path) was applied to the embedded tissue at intervals to ensure it remained frozen.
6. Once mounted onto the cryostat, the embedded tissue was sectioned with a stainless steel microtome blade (Feather S35). The sections were cut longitudinal to the tendon fibre, in thicknesses of either 5 or 8 µm.

7. The tissue sections were then mounted onto microscope slides and left at room temperature for 30 minutes to allow the cryo-embedding medium to evaporate before the sections were stained.

Figure 3.4: Biopsy samples from different aged volunteers

3.7 Staining of ex-vivo tissue

Haematoxylin and Eosin staining was used to stain the tissue for cells (cell nuclei appeared dark blue). It was important to characterise collagenous regions only and so areas of tissue rich in cellular structures could be identified by staining.

1. The section was washed in Formal Alcohol for 1 minute after which it was rinsed in water.

2. It was then stained in Gills Haematoxylin for 1 minute and then washed in water for 3 minutes.
3. It was then placed in Eosin for 30 seconds, differentiated in water, after which it was dehydrated, mounted onto a glass slide and covered with a cover slip.

Pico Sirius Red staining was used to stain the tissue for areas rich in collagen (collagenous regions appeared red).

1. Cryo-embedding medium was removed from the tissue by rinsing in Xylene.
2. The section was then washed in water for 10 minutes.
3. The section was then left to stain in Pico Sirius red for 30 minutes before being dehydrated rapidly in ethanol.
4. The section was then cleared in Xylene before being mounted onto a glass slide.
5. The glass slide was dried in air at room temperature, and was ready for AFM imaging.

3.8 **Histology of ex-vivo tissue**

Samples mounted on 76 x 22 mm (Agar Scientific LTD UK) microscope slides were imaged using a Lietz Leica optical microscope, using 10x, 20x and 30x objectives. Calibration was performed and scale bars added using ImagePro-Plus software (Media Cybernetics, USA).

3.9 **AFM tip calibration**

Tips required calibration in order to determine their sensitivity, spring constant and thus force applied. The sensitivity of a tip was obtained by applying a load to a hard substrate (glass microscope slide) to ensure laser deflection on the photodiode was purely a result of cantilever bending. The gradient of the resultant V/nm graph provided the sensitivity
of the tip to give how many volts of deflection corresponded to a nanometer of deflection. The thermal tune method was then employed so thermal oscillations of the cantilever could be related to spring constant using the equipartition theorem (Hutter and Bechhoefer 1993).

Sensitivity of a tip was obtained by applying 0.60 V vertical deflection from approach set-point to a glass substrate. The gradient of the retraction curve was fitted using JPK Nanowizard Control software to obtain a tip sensitivity. The tip was retracted from the surface by 30 µm before resonant frequency measured and peak fitted using JPK Nanowizard Control software to obtain the tip spring constant.

### 3.10 AFM Imaging

High resolution AFM imaging of samples was carried out in air, with soft probes. There were a variety of scan sizes used to identify noteworthy morphological features in the sample – from 5 µm to 20 µm. Proportional and Integral gains from the PI feedback loop were adjusted accordingly and the error signal of the feedback loop used for image analysis. All AFM images were acquired directly from the histological sections without any further processing or re-hydration.

AFM imaging was performed using a Nanowizard II AFM (JPK Instruments, Germany) system mounted on an Olympus IX71 (Olympus, Japan) inverted microscope. Imaging was performed in contact mode using MSNL-10 probes (Bruker, Santa Barbara, USA) with spring constants of 0.1-0.3 Nm⁻¹ at a scanning rate of 1.0 Hz or above.
**Dermis**

Four individual cryosections from each volunteer, mounted on microscope slides provided four distinct tissue samples from each volunteer. The sections were divided into four regions (all within the reticular dermis) where imaging was performed. Images were obtained with scan sizes of 5x5 $\mu$m$^2$, 10x10 $\mu$m$^2$ and 40x40 $\mu$m$^2$.

**Anterior tibialis and Achilles tendon**

Four individual cryosections from each volunteer, mounted on microscope slides provided four distinct tissue samples from each volunteer. The sections were divided into four regions (all within bulk tendon) where imaging was performed. Images were obtained with scan sizes of 5x5 $\mu$m$^2$, 10x10 $\mu$m$^2$ and 40x40 $\mu$m$^2$.

**3.11 AFM image analysis**

Images acquired by JPK Nanowizard Control software were imported into Gwyddion 2.37 open source scanning probe microscopy image analysis software (Gwyddion is supported by Department of Nanometrology, Czech Metrology Institute) whereupon image contrast could be adjusted and scale bars added. Qualitative analysis was carried out to identify changes to morphology indicating morphological biomarkers of ageing.

**3.12 AFM Nanoindentation**

Prior to indentation, the sample surface was imaged in order to identify fibrils for nanoindentation. Once an image was obtained, individual fibrils were selected for indentation, with location of loading specified as shown in figure 3.5. A loading regime was then applied and force-distance curves obtained.
Imaging and nanomechanics were performed in air, using RFESPA probes (Bruker, Santa Barbara, USA) with an approximate spring constant $k=3 \text{ Nm}^{-1}$, actual tip spring constant was found through calibration. Once collagen fibrils were identified, they were indented using a load of 300 nN with a tip extension and retraction speed of 2 $\mu \text{ms}^{-1}$.

### 3.13 Nanoindentation data analysis

Once the sample was indented locally on a point of interest, force-displacement (F-D) curves were obtained for both the extension and retraction parts of the indentation, an example shown in figure 3.6. The curves were then processed in JPK Image Processing Software (JPK Instruments, Germany) where baseline correction, in both x and y axes were performed and a tip-sample separation correction was carried out to obtain actual indentation depths. The curves were exported to .txt format where they could be processed using a custom MATLAB® script developed by the author (section 3.20) to calculate the Reduced Young's Modulus ($E$) of a sample using the Oliver-Pharr method.
The custom MATLAB® output a window to show curves prior to and after tip-sample separation correction, as well as the segment of interest of the F-D curve in question for fitting using Oliver-Pharr model. This bulk view of all curves fitted allowed a preview of analysis so anomalies or incorrect curve segment analysis could be spotted immediately.

![F-D curve obtained by JPK Data Processing software. Extend curve (blue) and retract curve (red).](image)

**3.14 AFM Single Molecule Force Spectroscopy (SMFS)**

The force-displacement curve of a single molecule pulling experiment is affected by a variety of factors which include contact force, time on surface and retraction rate. Contact force must be kept to a minimum to prevent indentation of the collagen fibril – this is performed by approaching with a low set-point and decreasing until a critical set-point is reached whereby the tip does not approach or remain on the sample surface. The time the tip is kept on the surface (dwelling time) is important to ensure adhesion between the tip and collagen molecules. The retraction rate is a more complicated parameter as altering this can impact on the number of unbinding events observed. A too slow or too
fast retraction rate results in unbinding events not being observed. To avoid biasing the data, both attraction and loading rates were kept constant for all samples.

The sample was hydrated with ultra-high quality (UHQ) water for a period of 10 minutes before submersion of the tip into the water droplet. The tip was then approached to the sample surface with an approximate set-point of 0.20 nN. Locations for force spectroscopy were selected and a loading regime applied. The load applied was minimal to ensure contact only and to prevent indentation. The tip approach speed was 1 µms⁻¹, the tip was held at contact with the sample for 4 seconds to promote molecular adhesion and then retracted at a speed of 1 µms⁻¹. The resultant F-D curves for approach and retract were saved and processed.

3.15 SMFS data analysis

F-D curves were imported into JPK Image Processing Software (JPK Instruments, Germany) where curves were fitted individually with the WLC model. Analysis was carried out on retraction curves and individual unbinding events were selected manually to ensure correct fitting. The persistent length was 14.5 nm in coalescence with Sun et al. 2002 (Sun, Luo et al. 2002).
3.16 Differential Scanning Calorimetry

Sample preparation of in-vitro collagen model

1. Rinse collagen gel in distilled and filtered water to remove PBS.
2. Dissect collagen gel into 5x5 mm square and allow to air dry for 30 minutes.
3. Place into aluminium crucible (previously weighed empty) and using tweezers cover the bottom of the crucible completely with the collagen sample, ensuring full contact.
4. Weigh the crucible with sample on scales to obtain the mass of the sample. Seal the crucible with a press, making it water-tight.
5. Apply the first regime, this includes a freezing following by a melting ramp (reaching denaturation temperature).
6. Perforate the crucible with a needle, being careful not to open it or disturb the sample within.
7. Apply the second regime, this includes a heating ramp for complete evaporation of loose water within the sample.

Experimental parameters

A Mettler Toledo DSC1 (Mettler-Toledo International Inc.) was used with aluminium crucibles. There were three heating regimes applied;

Sealed crucible

Ramp 1: 25°C to -30°C to allow freezing of water

Ramp 2: -30°C to 85°C to allow collagen denaturation, without evaporation of loosely bound interstitial water
Perforated crucible

Ramp 3: 25°C to 180°C to allow evaporation of loosely bound interstitial water

DSC data analysis

StarE Evaluation software used for analysing DSC curves to obtain onset temperature of collagen denaturation and calculate enthalpy of collagen denaturation.

3.17 Scanning electron microscopy

An XL-30 FEG SEM (Philips) was used for electron microscopy. Sample preparation was carried out as below;

1. The microscope slide containing the tissue section was sectioned using a diamond glass cutter. The sectioned slide was then sprayed with compressed air to remove particles of broken glass.
2. The sectioned slide was mounted onto an aluminium SEM specimen stub with an adhesive and this was left to dry for 2 hours at room temperature.
3. The stub and attached slide was then coated in a conductive gold layer using a sputter coater (Polaron equipment LTD E5000).

3.18 Transmission electron microscopy

A CM 12 TEM (Philips) was used for transmission electron microscopy. Sample preparation was carried out as below;

1. Tendon was dissected into approximately 4 mm (length) x 1 mm (diameter).
2. The tendon was dehydrated in graded ethanol solutions (20, 50, 70% for 15 minutes each followed by 90% thrice for 10 minutes each).

3. Once dehydrated, the tendon section was infiltrated with London Resin White (LR white) at a ratio of 1:1 with 90% concentrated ethanol) for 30 minutes followed by pure LR white solutions for 30 minutes, 24 hours and then 30 minutes.

4. This was then embedded by adding 22.5 µl of cold accelerator to tendon in 14 ml of LR white resin.

5. This was left to set at 4°C for 24 hours where it solidified.

3.19 Sampling

In-vitro tissue models were glycated for various durations individually, in sealed culture tubes, opened at end of glycation duration. Each time point had a separate sealed hydrogel, with all hydrogels in a cohort (e.g. glucose glycated) produced from the same master batch to ensure standardisation.

Each hydrogel was sectioned into 4 pieces (measuring 5 mm x 5 mm) before mounting on a glass slide. Each section was divided into four quadrants with high resolution AFM imaging conducted on each quadrant a minimum of 3 times. Nanoindentation of each quadrant was conducted with approximately 100 indentations per section, selecting manually fibrils to be indented. A high sampling size accounted for failed indentations. Pooling data from the 4 sections counteracted low sample sizes due to failed indentations. Single molecule pulling experiments were conducted on 2 sections per time point with 256 points selected per section, using a grid overlay.
Ex-vivo tissue samples were cryosectioned 4 times, with each section divided into 4 quadrants for high resolution imaging. Nanoindentation was conducted on 3 cryosections with approximately 100 indentations per section, with fibrils selected manually.

Single molecule pulling experiments were conducted on 2 sections per tissue type with 256 points selected per section, using a grid overlay.

3.20 Statistical analysis

Histograms were plotted using Origin® (OriginLab, USA). Statistical analysis was performed using SPSS Statistics (SPSS Inc, USA).

Young’s Modulus of fibrils was compared using two sided hypothesis testing with significance level $\alpha=0.001$. For non-paired comparison of two exclusive groups, two-tailed Student’s t-test was used (SPSS 22) with an F-test to check for equality of variance. For comparison within the exclusive groups, one-way ANOVA was used (SPSS 22). For comparison of non-parametric data, Kolmogorov–Smirnov test was used (OriginLab, USA). Standard error is used throughout each chapter.
% OLIVER PHARR with Tip sample ratio correction factor
% Written by Tarek Ahmed, PhD at UCL.
% Contact tarek.ahmed.10@ucl.ac.uk
% Refer to "Oliver-Pharr MATLAB seminar 27MAY16.ppt" for instructions
% Conditions for use:
% 1) You acknowledge me in papers/reports/posters/presentations
% 2) You do not modify the script without notifying me
% 3) You notify me of any publications that arise from the use of the script

%tip geometry
%region
%poisson's ratio

%------- SETTING PARAMETERS ---------------------
number_curves=300;
Filename='SAMPLE NAME';
percentage=0.25;
column_number_extend_uncorrected=1;
column_number_corrected=7;
column_number_retract_uncorrected=1;
column_number_retract_corrected=7;

%------ DO NOT ALTER BELOW THIS -----------
for i =1:number_curves;
    ivalue=int2str(i);
    f=[Filename,'extend ', '(', ivalue, ')', '.txt'];
    A{i}= importdata(f,' ',75);
    B{i}=[A{1,i}.data(:,column_number_extend_uncorrected),A{1,i}.data(:,2)];
    hold on
    subplot(3,2,1);
    plot(B{i}(:,1),B{i}(:,2));
    title('uncorrected extend');
    minforce_col=find(B{i}(:,2)==min(B{i}(:,2)));%
    min_force(i)=minforce_col(1);
    deflection
    min_force(i)=minforce_col(1);
    maxforce_col=find(B{i}(:,2)==max(B{i}(:,2)));
    max_force(i)=maxforce_col(1);
    x0(i)=B{i}((max_force(i)),1);
    x1(i)=B{i}((min_force(i)),1);
    delta_uncorrected(i)=x0(i)-x1(i);
    %h_uncorrected(i)=delta(i);
    %F(i)=max(B{i}(:,2));
    %grad(i)=F(i)/delta(i);
    hold on
    %define value range for m (optimise or percentage)
    subplot(3,2,2);
    plot(B{i}(min_force(i):max_force(i),1),B{i}(min_force(i):max_force(i),2));
    title('uncorrected extend sectioned');
end

for i =1:number_curves;
    ivalue=int2str(i);
    f=[Filename,'extend ', '(', ivalue, ')', '.txt'];
A{i} = importdata(f, ' ', 75);
B{i} = [A{i}.data(:, column_number_extend_corrected), A{i}.data(:, 2)];
hold on
subplot(3, 2, 3);
plot(B{i}(:, 1), B{i}(:, 2));
title('corrected extend');
% element
minforce_col = find(B{i}(:, 2) == min(B{i}(:, 2)));  
min_force{i} = minforce_col(1);  
% not actual - element
maxforce_col = find(B{i}(:, 2) == max(B{i}(:, 2)));  
max_force{i} = maxforce_col(1);  
% deflection
x1{i} = B{i}((min_force{i}), 1);  
% FIND Pmax
x0{i} = B{i}((max_force{i}), 1);%
figure
plot(x0{i}, x1{i});
delta_corrected{i} = x0{i} - x1{i};  
% h_corrected{i} = delta{i};  
h2 = delta_corrected';

F{i} = max(B{i}(:, 2));
grad{i} = F{i}/delta{i};
hold on
% define value range for m (optimise or percentage)
subplot(3, 2, 4);
plot(B{i}(min_force{i}:max_force{i}, 1), B{i}(min_force{i}:max_force{i}, 2));
title('corrected extend sectioned');
end

for i = 1: number_curves;
ivalue = int2str(i);
f = [Filename, 'retract ', '(', ivalue, ' ', '.txt'];
A{i} = importdata(f, ' ', 75);
B{i} = [A{i}.data(:, column_number_retract_corrected), A{i}.data(:, 2)];

subplot(3, 2, 5);
plot(B{i}(:, 1), B{i}(:, 2));
title('corrected retract');
hold on;
maxforce_col = find(B{i}(:, 2) == max(B{i}(:, 2)));  
max_force{i} = maxforce_col(1);  
% element
minforce_col = find(B{i}(:, 2) == min(B{i}(:, 2)));  
min_force{i} = minforce_col(1);  
% not actual - element
maxforce_col = find(B{i}(:, 2) == max(B{i}(:, 2)));  
max_force{i} = maxforce_col(1);  
% deflection
x1{i} = B{i}((min_force{i}), 1);  
% FIND Pmax
x0{i} = min(B{i}(:, 1));
delta{i} = (x0{i} - x1{i});
delta_new{i} = delta{i};
F{i} = max(B{i}(:, 2)) - min(B{i}(:, 2));
indent_range{i} = abs(max_force{i} - min_force{i});
gradbegin(i)=round(percentage*indent_range(i));

F_1(i)=(B(i)(gradbegin(i),2));

x_1(i)=(B(i)(gradbegin(i),1));
x_0(i)=(B(i)(1,1));

poly_fit{i}=[polyfit((B{i}(1:gradbegin(i),1)),(B{i}(1:gradbegin(i),2)),1)];
gradd_fitted(i)=abs(poly_fit{i}(1,1));
gradd_calculated(i)=((F(i)-F_1(i))/(x_1(i)-x_0(i)));
gradd_uncorrected(i)=abs(F(i)/delta(i));
gradd_corrected(i)=abs(F(i)/delta_new(i));

hold on
%define value range for m (optimise or percentage)
plot(3,2,6);
plot(B{i}(1:min_force(i),1),B{i}(1:min_force(i),2));
plot(B{i}((1:gradbegin(i)),1),(B{i}((1:gradbegin(i)),2)));
title('corrected retract sectioned');
hold on;

%polyfit_m(i)=-(poly_fit{1,i}(1,1));
gradd_used(i)=grad_fitted(i);
epsilon=0.75;
h_max(i)=abs(delta_new(i));
h_s(i)=(abs((epsilon)*(F(i)/grad_used(i))));
h_c(i)=abs(h_max(i)-h_s(i));
R=4e-9;
A_hc(i)=abs(-3.1416*((h_c(i))^2)+(2*3.1416*R*(h_c(i))));
v=0.5;
alpha=1.2304*(1-0.21*v-0.01*(v^2)-0.41*(v^3));
um_eq(i)=grad_used(i)*(sqrt(3.1416));
den_eq(i)=2*sqrt(A_hc(i))*alpha;
Eop_row(i)=num_eq(i)/den_eq(i);
Eop_column=Eop_row';
EopMPa_row(i)=Eop_row(i)/1000000;
EopMPa_row(i)=Eop_row(i)/1000000000;
EopGPa_column=EopGPa_row';

%%E_row{i}=[(poly_fit{i}(1,1)/delta(i))*3.74];
%%EMPa_row(i)=E_row{i}/550000;
%%EMPa_column=EMPa_row';
%disp('All E values for Uncrosslinked FD5')
end
disp('-----Youngs Modulus calculated using Oliver-Pharr model');
disp(Filename);
disp('Open Eop_column in Workspace to view calculated vales (in Pa)');
disp('View the graphs to see which part of the curves have been used for calculations')

%h=[h1 h2 h3];
4 Development of Studies

4.1 Aim of the chapter

This chapter aims to investigate specifically the change in biophysical and structural properties of collagen as a function of glycation. This requires localisation of the effects of cross-linking on collagenous tissue. This can be best achieved through in-vitro techniques glycating agents can be introduced in a controlled manner to initiate AGE cross-linking. By controlling the cross-linking agents present, the effect of AGE cross-links can be separated out from other biochemical changes to collagen in the in-vitro glycated collagen tissue model. Changes to structural properties at the nanoscale can be investigated using a suite of techniques characterising collagen at the fibrillar scale.

The aim above can be divided into the following two objectives;

**Develop an in-vitro model**

A suitable in-vitro glycated collagen tissue model must be produced. There are a variety of techniques to produce in-vitro collagen models. It is important to be able to modify collagen models in a systematic manner for controlled glycation. Upon successful glycation, the models must be applicable for characterisation.

**Investigating possible morphological and nanomechanical changes as function of AGE cross-linking**

The in-vitro glycated tissue models can then be characterised to look at two specific areas of change; morphological (changes to ultrastructure) and mechanical (changes to mechanical and structural properties). The impact of AGE cross-linking must be investigated using a series of techniques sensitive enough to characterise biophysical and
structural changes brought about by AGE accumulation. The techniques must be applicable in environments analogous to native collagen tissue environment to ensure accurate representation of in-vivo biomechanical properties.

Because of the depth of the work presented, we can separate the work carried out into two stages 4a (Developing the model and understanding morphology of collagen), and 4b (Investigating nanoscale mechanical properties of in-vitro tissue models as function of glycation)

a) Developing the model and understanding morphology of collagen

4.2 Developing an in-vitro model

4.2.1 In-vitro methods

There are two commonly used methods for producing an in-vitro model of collagen matrix. The plastic compression method developed by Wiseman (Brown, Wiseman et al. 2005) and the RAFT method.

Plastic compression (PC)

A method to develop 3D tissue models with a similar collagen density as various native tissues. This is the method of controlled and rapid plastic compression (PC) to produce acellular collagen scaffolds with mature quaternary collagen structure and relies on the rapid removal of interstitial water resulting in a reduction in water content from 99% to 85-90% (Cheema and Brown 2013). This leaves dense, collagenous tissue models with a
comparable density and water content level to native tissue. The in-vitro tissue models, often referred to as collagen sheets are approximately 50 µm in thickness and are ready for further processing to produce in-vitro glycated tissue models.

**Real Architecture for 3D Tissues (RAFT)**

An updated method utilising the principles of the PC method was developed to produce a consistent, time efficient way of making reproducible tissue models with uniformity in properties such as hydration, thickness and shape. This method relies on removal of interstitial water in the collagen hydrogel by capillary action through the use of stabilisers. The resultant in-vitro tissue model is a thin, dense and uniform collagen scaffold approximately 60-200µm thick (thickness depending upon the size of the well and stabilisation time). The RAFT method proved to be suitable for this study as it proved to be more efficient to produce a high volume of standardised collagen models in a short span of time.

4.2.2 Finding the right pH

There have been a number of studies investigating the effect of pH and electrostatic interactions on fibrillogenesis (Cassel 1966, Cooper 1970, Bard and Chapman 1973, Hayashi and Nagai 1974). It is evident that in-vitro fibrillogenesis can occur in a range of pH and to achieve optimal fibril formation requires a measure of control of these conditions as shown in an in-vitro study by Li et al (Li, Asadi et al. 2009). Li investigated the effect of electrostatic interactions on fibrillogenesis found the rate of fibrillogenesis significantly slowed down at lower pH, leading to the formation of heterogeneous fibrils,
ranging in diameter and with a high occurrence of small diameter fibrils (mean diameters of 85 nm at pH 6.6 and 200 nm at pH 8.0) (Li, Asadi et al. 2009). The characteristic D-banding was also affected, showing the impact on molecular aggregation and stacking.

As discussed in 2.1.1, in-vitro fibrillogenesis can occur once collagen monomer solution (solubilised in acetic acid) is neutralised with NaOH as described in both the PC and RAFT methods. The pH indicator used conventionally in both PC and RAFT methods is phenol red, contained in Minimum Essential Medium (MEM) added to collagen solution as described in the methods section. The pH of hydrogels produced using phenol red is 8.2. In order to identify the optimal pH for producing collagen hydrogels, a series of hydrogels were produced at slightly differing pH. Gels were produced with i) MEM containing phenol red ii) phenol red with no culture medium iii) bromothymol blue with no culture medium. Bromothymol blue undergoes a colour change to deep blue at pH 7.6, allowing collagen hydrogels to be produced at closer to neutral pH. Once neutralised, the tissue models were produced using the RAFT method and imaged using AFM to visualise fibril morphology. Gels produced using phenol red and bromothymol blue as indicators are shown in figure 4.1.

Figure 4.1: Collagen hydrogel produced using phenol red as pH indicator (a) and bromothymol blue as pH indicator (b).
4.2.3 Neutralisation of collagen gels

The hydrogel produced using MEM (pH 8.2 with culture medium), successful fibril formation is achieved as shown in figure 4.2(a) with homogeneous fibril thickness and the characteristic d-banding denoting the formation of mature fibrils (*). While fibril formation (*) can be seen to have occurred in figure 4.2(b), hydrogel produced with just phenol red (pH 8.2 without culture medium), there is some heterogeneity among their structure and a partial absence of D-banding signifies immature fibril formation (**). This is seen to a greater extent (**) with the use of bromothymol blue in figure 4.2(c) - hydrogel with pH 7.6 and without culture medium, along with areas of collagen hydrogel showing no fibrillar structure (***)

The effect of pH on fibrillogenesis is clear and in line with the study by Li et al (pH 8.2 formed higher quality hydrogels than pH 7.2). However, the difference in fibril morphology shown between hydrogel produced with MEM figure 4.2(b), and hydrogel produced with just phenol red figure 4.2(b), both set at pH 8.2, alludes to an altering effect on fibrillogenesis by the absence of culture medium. This may be explained by the increased ion content provided by the culture medium in MEM. As described by Mertz and Leikin (Leikin, Rau et al. 1995), there are a number of groups found in residues which are ionisable and affect electrostatic interactions of collagen molecules during fibrillogenesis.

Effective in-vitro fibrillogenesis may require the presence of ions in the surrounding environment to regulate electrostatic interactions. As we know, there is an added complexity of fibrillogenesis in-vivo with the need for fibronectin and integrin regulating the process (Kadler, Hill et al. 2008). This implies that though fibrillogenesis is an entropic process, it can be regulated by further components, such as the surrounding
90 ionic concentration in the case of in-vitro fibrillogenesis. It was therefore concluded that producing hydrogels using MEM was effective in consistently forming high quality in-

Figure 4.2: Representative AFM images of in-vitro collagen hydrogel produced using a) MEM containing Phenol red, b) Phenol red with no culture medium, c) Bromothymol blue with no culture medium. * marks areas displaying fibril formation, ** marks absence of banding structure, *** marks areas of no fibrillar structure

4.2.4 Glycating agents for the in-vitro glycated tissue models

Glycation is initiated by the reaction of aldoses with residues (i.e. lysine) in the collagen molecule. As the C=O bond from glucose reacts with the amine group of the lysine, it undergoes numerous reversible steps to form a Schiff Base, followed by subsequent reversible steps to form the Amadori product and then glucosepane. The reversibility of various mechanisms in the process as well as the need for glucose to form dextrose (d-glucose, its open chain form) for initiation means the reaction rate is slow. This is the underlying reason why concentration of glucosepane and other AGE products are found to be substantially higher in tissue with a low collagen turnover (Verzijl, DeGroot et al. 2000).

In-vitro glycated tissue model: Glucosepane cross-linked

It was important to produce in-vitro glycated models mimicking the accumulation of glucosepane mediated by glucose. However, due to the slow nature of the Maillard
reaction, producing in-vitro glycate model crosslinked with a substantial concentration of glucosepane would take a significant length of time. After multiple trials of creating glucose glycated models, it was found to be necessary to produce in-vitro glycate tissue models at a faster glycation rate.

**In-vitro glycated tissue model: GOLD and MOLD cross-linked**

In-vitro glycated tissue models with a various different AGE cross-linking were produced. It was possible to “skip steps” in the Maillard reaction by initiating glycation with degradation compounds. This included glycation mediated by glyoxal and methyglyoxal. In-vitro glycated tissue models produced using these agents however would result in the accumulation of other AGE cross-links as shown in figure 1.8, in this case GOLD (glyoxal lysine dimer) by using glyoxal and MOLD (methylglyoxal lysine dimer) by using methyglyoxal. The relative concentrations of these AGEs in-vivo are far lower than glucosepane (Avery and Bailey 2005, Monnier, Sun et al. 2014).

4.2.5 Concentration of glycating agents

The concentration of the various glycating agents vary greatly in-vivo. Glucose is the most common sugar with an in-vivo plasma concentration of approximately 5.6 mM (Danaei, Finucane et al. 2011, Liao, Tu et al. 2015) (fasting). The plasma concentrations of glyoxal and methyglyoxal are more difficult to measure, with early estimates being in the order of tens of mM in the case of methyglyoxal (Chaplen, Fahl et al. 1996, Kalapos 1999). More recent estimates of in-vivo plasma concentrations of glyoxal and methyglyoxal are approximately 100 nM (Strzinek, Scholes et al. 1972, Beisswenger, Howell et al. 1999) (both) with estimates of cellular concentration being 0.1-1 µM for glyoxal and 1-5 µM for methyglyoxal (Dobler, Ahmed et al. 2006). Previous studies have successfully achieved
in-vitro glycation of various proteins such as human albumin serum over the course of 3 weeks with 50 mM glucose, incubated at 37°C for 3 weeks at pH 7.4. Another study initiating in-vitro glycation incubated rabbit tendon in 133 mM glucose at 35°C over a course of 3 weeks also reported the presence of AGE products (Kent, Light et al. 1985).

There have been studies which have used in-vitro collagenous tissue models as a substrate for producing glyoxal and methyglyoxal mediated AGEs. One such study glycated a collagen-chitosan substrate with 500 µm glyoxal for a period of 31 days and measured an increase of CML expression (Cadau, Leoty-Okombi et al. 2015). Another study investigating effect of glyoxal on fibroblast activity incubated cells with 1 mM glyoxal for a period of 20 hours (Knels, Valtink et al. 2012). A study which measured the effects of selectively glycating reconstituted in-vitro models of skin with different glycating agents incubated the in-vitro tissue model with 1 mM methylglyoxal for a period of 24 hours (Pageon, Zucchi et al. 2015).

4.2.6 Concentration assay for glycating agents

In order to accelerate the formation and accumulation of AGE cross-links, an assay was performed with various concentrations of glycating agents (buffered in PBS), incubating for various periods of time. Glycation was initiated post neutralisation, to an already formed hydrogel as this more accurately simulates the physiological conditions of in-vivo glycation.

It is known that in-vitro glycation of collagen results in a discolouration of collagen, in the form of “browning” and visible to the naked eye (Liang and Rossi 1990). Visual changes in the in-vitro models could be used as a means of assessing whether glycation was successful. An example of such changes are shown in figure 4.3. The image of the glycated gel (right) illustrates the browning effect.
An initial assay was performed to understand the impact of concentration on the tissue model. This started at similar concentrations used in literature until the highest concentration that could be used without deleterious effects on the tissue model. These concentrations would be pathological for cells, however this was not the subject of the study. A second, time based assay was then performed at the selected concentration to investigate the impact of the accumulated AGEs on the biophysical properties of the in-vitro glycated tissue models. The results of assays are shown in the tables below.

### Glucose

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>1 day</th>
<th>3 days</th>
<th>7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>No change</td>
<td>No change</td>
<td>No change</td>
</tr>
<tr>
<td>10</td>
<td>No change</td>
<td>No change</td>
<td>No change</td>
</tr>
<tr>
<td>50</td>
<td>No change</td>
<td>No change</td>
<td>No change</td>
</tr>
<tr>
<td>100</td>
<td>No change</td>
<td>No change</td>
<td>No change</td>
</tr>
<tr>
<td>200</td>
<td>No change</td>
<td>No change</td>
<td>No change</td>
</tr>
<tr>
<td>722</td>
<td>No change</td>
<td>No change</td>
<td>No change</td>
</tr>
</tbody>
</table>

Table 2.1: Incubation time required to see a visible change in glucose glycated tissue model

After incubation with various concentrations of glucose, no visual change could be observed with the naked eyes. Due to the slow nature of glucosepane formation, it was imperative to glycating tissue models using glucose at the highest concentration possible. D-glucose was therefore added to PBS at the maximum solubility concentration, 722 mM
glucose. Visual changes could not be observed after 1 week of incubation at 37°C, however this concentration was selected for further experiments.

**Glyoxal**

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>1 day</th>
<th>3 days</th>
<th>7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>No change</td>
<td>No change</td>
<td>No change</td>
</tr>
<tr>
<td>100</td>
<td>No change</td>
<td>No change</td>
<td>No change</td>
</tr>
<tr>
<td>200</td>
<td>No change</td>
<td>No change</td>
<td>No change</td>
</tr>
<tr>
<td>400</td>
<td>No change</td>
<td>No change</td>
<td>No change</td>
</tr>
<tr>
<td>800</td>
<td>No change</td>
<td>No change</td>
<td><strong>Browning</strong></td>
</tr>
<tr>
<td>1250</td>
<td>No change</td>
<td><strong>Dissolved</strong></td>
<td><strong>Dissolved</strong></td>
</tr>
</tbody>
</table>

Table 2.2: Incubation time required to see a visible change in glyoxal glycated tissue model

The highest concentration of glyoxal in PBS found to be suitable for incubation was 800 mM. Less concentrated solutions did not exhibit visual changes to the tissue model, while higher concentrations led to rapid breakdown of the hydrogel structure.

**Methylglyoxal**

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>1 day</th>
<th>3 days</th>
<th>7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>No change</td>
<td>No change</td>
<td><strong>Browning</strong></td>
</tr>
<tr>
<td>100</td>
<td>No change</td>
<td>No change</td>
<td><strong>Browning</strong></td>
</tr>
<tr>
<td>200</td>
<td><strong>Browning</strong></td>
<td><strong>Dissolved</strong></td>
<td></td>
</tr>
<tr>
<td>400</td>
<td><strong>Dissolved</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.3: Incubation time required to see a visible change in methylglyoxal glycated tissue model
In the case of methylglyoxal, rapid breakdown of the hydrogel could be observed at far lower concentrations relative to glyoxal. The highest concentration found to be suitable for incubation was 100 mM.

4.2.7 Nanoscale morphology of native collagen

Once in-vitro glycated tissue models have been formed, changes in collagen nanoscale morphology can be observed as a function of glycation. Fibril morphology may be thought of as a marker for fundamental fibrillar properties. The overall structure of collagenous tissue may remain visually unchanged at the macroscale, however be significantly different at the nanoscale. It is important to establish a benchmark of collagen nanoscale morphology. Presented in figure 4.4 are representative AFM images of rat tail tendon (a) and rabbit tendon (b). The morphology of collagen fibrils can be ascertained from analysing these images.

Figure 4.4: a) Native collagen fibrils from rat tail tendon (Bozec and Odlyha 2011), b) native collagen fibrils from rabbit tendon. * marks fibrils with clear diameter, ** marks fibrillar banding, *** marks strong alignment of fibrils
While it is clear from figure 4.4 that there are differences in nanoscale morphology between various collagenous tissues, there are common characteristics which are described below.

**Fibril diameter (*)**: Collagen fibrils have a distinct tube-like shape with a consistent diameter once fully formed. A collagen matrix with collagen fibrils sharing similar diameters is indicative of mature and homogenous fibril formation.

**Fibril d-banding (**)**: The banding pattern observed along fibrils is characteristic of type-1 collagen fibrils. As described in the following chapter, it arises due to molecular arrangement.

**Fibril packing and arrangement (***)**: The packing and arrangement of collagen fibrils is also an important feature of consideration when analysing nanoscale collagen morphology. Fig. 4.4(b) shows the high degree of alignment of collagen fibrils typical of tendon tissue.

### 4.2.8 Indentation load

It is important for the indentation load to be such that the indentation depth of the tip is in the range of 5-10% of the collagen fibril diameter, to ensure the substrate surface (glass) mechanical properties do not overtly impact cantilever deflection. In a pilot study, a variety of indentation loads were applied to collagen fibrils and indentation depth measured (n=30) after applying tip-sample correction as described in the method section, mean depths are listed in table 2.4.
Table 2.4: Indentation depths as function of load

<table>
<thead>
<tr>
<th>Indentation load (nN)</th>
<th>Indentation depth (nm)</th>
<th>% of fibril diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>2</td>
<td>3%</td>
</tr>
<tr>
<td>50</td>
<td>2</td>
<td>3%</td>
</tr>
<tr>
<td>80</td>
<td>3</td>
<td>4%</td>
</tr>
<tr>
<td>300</td>
<td>6</td>
<td>8%</td>
</tr>
</tbody>
</table>

As shown in table 2.4, loads below 300 nN were found to result in a very low indentation depth (<5% of the average diameter size – see Appendix 1.1), leading to uncertainty as to whether the mechanical properties of the collagen fibril were accurately being tested. It was decided that a load of 300 nN as applied by Wenger et al. (Wenger, Bozec et al. 2007) (on rat tail tendon collagen fibrils) was most appropriate and therefore was selected for further experiments.

4.3 Results and Discussion

4.3.1 Identifying morphological markers of ageing in in-vitro glycated tissue models

The morphology of in-vitro glycated tissue models will be assessed in the following section with discussion about the distinction between changes in morphology brought about by other factors other than AGE cross-linking.

4.3.1.1 Breakdown of tissue model

A significant factor that was observed during incubation of the tissue models was the breakdown or dissociation of the tissue model matrix. Due to the weakly bound nature of collagen molecules in uncross-linked tissue models, this effect was more pronounced in uncross-linked samples. This breakdown progressed in significance from 4-5 weeks of
incubation onwards (with 8/10 images exhibiting this). Tissue models cross-linked in the presence of glucose, glyoxal and methylglyoxal also displayed this breakdown, however this was counteracted in some effect by cross-link formation. In the case of glucose however, due to the slow nature of the reaction, the breakdown effect was severe. Ultimately this meant there are two opposing factors affecting fibril morphology - breakdown of the matrix and cross-link formation. Below are examples of collagen fibril morphology at various incubation times, illustrating the breakdown of the collagen gel matrix. Characteristics displayed by breakdown of the gel are shown in Figures 4.5, 4.6, 4.7 and 4.8.

Control gels prior to incubation display well defined fibrillar structure as shown in Figure 4.5(a). Collagen fibrils are uniform in thickness and mature fibrillogenesis can be confirmed by the clear D-banding periodicity of 66.0±1.5 nm (N=30) observed.

Upon incubation, the fibrillar structure is slowly lost due to the loose affinity of collagen molecules. This manifests in areas of the gel where fibrils can be observed to be breaking down into constituent smaller fibrils as shown (*) in Figure 4.5(b). The d-banding periodicity is slowly lost as the collagen fibrils lose their quaternary structure.

After a long period of incubation (over 5 weeks), most of the collagen fibrils observed have lost their structural integrity as displayed (**) in Figure 4.5(c), breaking down to leave a gelatinous matrix. For gels incubated for over 5 weeks, the likelihood of imaging areas with a fully broken down matrix exceeds 50% (N=30).
Gels incubated with glucose also displayed similar signs of structural deterioration. In this case however, it was seen to be highly regional, with many areas displaying fully intact fibril structure after numerous weeks of incubation. This is illustrated by comparing Figure 4.6(a) which shows near complete breakdown of fibrillar structure after 5 weeks of incubation (*) and Figure 4.6(b) which shows little to no breakdown of fibrillar structure after 6 weeks of incubation (**).

This resilience of matrix integrity was more commonly observed in gels incubated with glucose than control gels. The likelihood of imaging areas with a fully broken down matrix after 5 weeks of incubation was less than 30% (N=57). Figure 4.6(c) is representative of matrix structure after a prolonged period of incubation. In this case, remnants of fibrillar structure can be observed (***) however this is mostly in the process of dissociation. There is a clear difference between the level of degeneration of the matrix when compared to the control (Figure 4.5).
Figure 4.6: Gel incubated with glucose for 35 days (a), 42 days (b) and 70 days (c). * marks fibrils with small diameter, ** marks fibrils with loss of structural integrity, *** marks remnants of fibrillar structure in an otherwise dissociated matrix.
The severity of deterioration of gels incubated with methylglyoxal was far lower than that of the control gels and glucose glycated gels. While localised areas of fibril dissociation (*) could be observed in AFM imaging (shown in Figure 4.7), the structure of the matrix was predominantly fibrillar during the 10 week period.

Similar deterioration characteristics were observed for gels incubated with glyoxal too. The extent of gel breakdown was far lower than that of the control gels and glucose glycated gels. Localised areas of fibril dissociation (*) were common as shown in AFM imaging (shown in Figure 4.8 – the bottom of the image shows the gelatinised form of collagen).
While control and glucose glycated gels exhibited almost complete breakdown in matrix structure from 5 weeks of incubation, glyoxal and methylglyoxal glycated gels exhibited matrix structures which were predominantly fibrillar during the 10 week period. This can be attributed to the faster rate of glycation of the latter two, resulting in significant levels of cross-link formation between fibrillar collagen before the fibril structure dissociates.

4.3.1.2 Morphological markers of various glycated tissue models

Although not discussed in the previous section, several unique morphological features could be observed in the AFM images of various glycated gels. The morphology of collagen fibrils without any glycation as shown in Figure 4.5(a) can be used as a baseline reference when discussing the changes brought on by glycation. As discussed in the previous section, gels glycated with glyoxal and methylglyoxal underwent cross-linking successfully, manifesting in an increase in the lifespan of the matrix structure. The analysis will therefore begin with these gels as these indicate the effects of glycation clearly.
Glyoxal glycated tissue model

Fibrillar alignment becomes pronounced in gels incubated with glyoxal for 56 days, as shown in Figure 4.9 (c) with fibrils seen to be mature and remain fully formed (*). The manifestation of fibril bundling can be observed in gels incubated for 70 days (**), displayed in Figure 4.9 (d). The tight winding of 3 or more fibrils to form larger scale bundles of fibrils is common. This interesting feature is not observed in control gels at all and may provide an indication as to whether cross-links formed are inter or intra fibrillar.

Figure 4.9: Gels incubated with glyoxal for 7 days (a) and 35 days (b), 56 days (c) and 70 days (d). * marks fully formed fibrils, ** marks fibril bundling

[Images of gels with labeled features]
**Methylglyoxal glycated tissue model**

Gels incubated with methyl glyoxal also exhibited clear morphological changes as shown in figure 4.10. Significant aligning and register along fibrils (*) can be seen after 14 days of incubation (a). Bundling of fibrils is also very common, with very few individual fibrils observable.

Such morphological features are further exacerbated in gels incubated for 42 days as shown in figure 4.10 (b). The severity of fibril bundling is heightened (**), with register (***) so pronounced, there is difficulty identifying individual fibrils in incoming bundles. It is interesting to note that the fibril register conserves the d-banding periodicity.

The fibril bundling does not seem to become more pronounced at later time points, alluding to a plateau being reached of AGE impact on morphology. Similar features of fibril bundling (**), register and alignment (*) are observed for gels incubated in methylglyoxal after 56 days (c) and 70 days (d). Fibrils retain their structure and d-banding periodicity.
Glucose glycated tissue model

For gels incubated with glucose, fibril morphology was found to be altered in gels incubated for a significantly long period of time compared with the other glycatin agents. From 6 weeks of incubation onwards, most regions displayed dissociated structure as described in the previous section. Remaining fibrils however were found to have altered morphology. Figure 4.11 (a) and (b) are representative images of altered fibril morphology from glucose incubation (*). Whilst fibril bundling is apparent as with...
methylglyoxal and glyoxal incubated gels (shown in Figure 4.11 – a), a unique feature observed was large scale register of innumerous fibrils, resulting in sheet like patterns (**) (shown in Figure 4.11 – b).

4.3.2 Summary of morphological markers

To summarise the morphological changes undergone by collagen at the fibrillar scale with respect to glycation, we can consider features observed in the glycated models that differentiated them from the control models. The control model was composed of gels containing fully formed mature fibrils with random orientation. Fibrils were mostly packed individually. Samples incubated with glucose, glyoxal and methylglyoxal showed signs of fibril bundling as well as some localised alignment. The fibrils were mostly mature with D-banding present. The bundling was found to increase significantly incubation, resulting in increased localised alignment.
b) Investigating nanoscale mechanical properties of in-vitro tissue models as function of glycation

Having examined morphological features, the chapter will now focus on the mechanical impact of AGE accumulation in the in-vitro models.

4.3.3 Identifying nanomechanical markers of glycated tissue models

4.3.3.1 Glucose glycated in-vitro tissue models

Nanoindentation

The nanomechanical characteristics of collagen fibrils investigated using the nanoindentation method presented a series of elastic modulus (E) measurements for each fibril indented. A comparison of the populations of E for various glucose glycated samples shown in figure 4.12 illustrate a change in distribution of E between control in-vitro scaffolds and scaffolds glycated with glucose for various periods of time. Starting with the control gel, it is observed that the interquartile range of E lies between 2.5 to 10 GPa with an average of 6.59±0.33 GPa. Measurements of elastic modulus for the subsequent weeks (week 1 and 2) do not show statistically significant changes (two-tailed Student's t-test as described in section 3.20). In average magnitude of E; E_{week1}=5.86±0.39 GPa (p=0.12), E_{week2}=7.13±0.56 GPa (0.40), as measured with the Kolmogorov-Smirnov test with p=0.05. However at week 5 a statistically significant decrease in E is observed E_{week5}=5.98±0.52 GPa (p=0.009). This is followed by no statistical difference observed between control and week 6 with E_{week6}=6.29±0.39 GPa (p=0.11). The statistically significant decrease of E at week 5 is then countered by a statistically significant increase in E at week 7 with E_{week7}=7.22±0.33 GPa (p=0.003). In-
vitro models incubated for longer periods of time then exhibit a statistically significant
decrease in $E$ in subsequent weeks; $E_{\text{week } 8}=3.68\pm0.32 \text{ GPa (p<0.001)}$, $E_{\text{week } 9}=3.57\pm0.23 \text{ GPa (p<0.001)}$ and $E_{\text{week } 10}=2.51\pm0.15 \text{ GPa (p<0.001)}$.

Figure 4.12: Boxplots of in-vitro collagen models glycated with glucose. Boxplot borders show the interquartile range (25% to 75% of data) with arms showing a range from 10% to 90% of the data. The median and mean are depicted by the central line and dot respectively.

Upon investigation of the distributions of $E$ for the various samples (figure 4.13), it is interesting to note the lack of normality of the control sample and the earlier time points. The measurements are varied and span a wide range of elastic modulus. Interestingly, after an initial tendency to skew towards lower $E$ observed at week 5, the distributions revert back to being wide and varied at week 6 similar in characteristics to a control gel, with a large proportion of high $E$ measurements at week 7 marking an increase in $E$, followed by a sudden reduction of $E$ measured resulting in tighter distributions skewed towards lower $E$, at weeks 8, 9 and 10, similar to the trend observed in figure 4.12 and supported by statistical analysis.
Figure 4.13: Stacked histograms showing elastic modulus (bin size 1 GPa) of in-vitro collagen models glycated with glucose for various periods of time.
Investigation of the contour lengths measurements for glucose glycated in-vitro models yield interesting results. Boxplots shown in Figure 4.14 show a difference between control model and glucose glycated models at subsequent weeks. The mean contour length ($L_0$) of a control in-vitro model was found to be $L_{0,\text{control}}=120.2\pm5.8$ nm. This was found to undergo a statistically significant decrease after 1 week of glycation to $L_{0,\text{week1}}=101.50\pm2.25$ nm ($p=0.008$). Interestingly, there was then a statistically significant increase in $L_0$ in the following weeks with a mean contour lengths of $149.28\pm3.87$ nm (week 4) and $146.92\pm5.25$ nm (week 6) followed by a statistically significant decrease in contour length.

Assessing the distributions of $L_0$ in figure 4.15, stacked histograms show a clear skew towards lower $L_0$ from control model to week 1. Interestingly, as with nanoindentation (figure 4.13), measurements of contour length on the control model shows no clear
parametric distribution, with data spanning a variety of values. The distributions of weeks 4 and 6 clearly show an increase in L_0 before a final decrease in weeks 8 and 10.

Figure 4.15: Stacked histograms of contour length (bin size 10 nm) of in-vitro collagen models glycated with glucose for various periods of time.
4.3.3.2 Glyoxal glycated in-vitro tissue models

Nanoindentation

Upon comparison of measurements of $E$ from glyoxal glycated in-vitro tissue models presented in Figure 4.16, an overall decrease in the median of $E$ can be observed with increased incubation time. The control in-vitro model had a mean $E$ of $6.59\pm0.34$ GPa and while the sample incubated for 1 week had a mean $E$ of $5.91\pm0.34$ GPa, this difference was not statistically significant using the Kolmogorov-Smirnov test ($p=0.16$). The following samples incubated with glyoxal for longer periods of time were found to be significantly different to the control sample with means of $E_{\text{week}2}=5.13\pm0.23$ GPa ($p<0.001$), $E_{\text{week}4}=3.26\pm0.26$ GPa ($p<0.001$), $E_{\text{week}8}=5.22\pm0.21$ GPa ($p<0.001$) and $E_{\text{week}10}=4.12\pm0.35$ GPa ($p<0.001$). The decrease in $E$ at week 4 followed by a slight increase in week 8 is statistically significant, however the difference between week 4 and week 8 is not ($p=0.08$).

Figure 4.16: Boxplots showing elastic modulus measurements ($E$) of in-vitro collagen models glycated with glyoxal for various periods of time.
The distribution of E (figure 4.17) show an interesting development as the glycation period progresses. The distributions of E for a control and in-vitro model incubated for a week are non-parametric, spanning a wide range of values. This then changes in further weeks as the distributions become centred around an average value and skewed towards lower E.

Figure 4.17: Stacked histograms showing elastic modulus (bin size 1 GPa) of in-vitro collagen models glycated with glyoxal for various periods of time.
SMFS

Contour length measurements of glyoxal glycated in-vitro models show an immediate decrease after glycation (figure 4.18). The contour length for a control in-vitro model was $L_{0,\text{control}}=120\pm5.84$ nm. This was found to decrease to $L_{0,\text{week2}}=61.48\pm3.22$ nm (p<0.001), $L_{0,\text{week4}}=52.95\pm2.42$ nm (p<0.001), $L_{0,\text{week8}}=60.07\pm4.69$ nm (p<0.001) and $L_{0,\text{week10}}=59.45\pm8.81$ nm (p<0.001) at subsequent weeks of glycation.

![Boxplots showing contour length measurements of in-vitro collagen models glycated with glyoxal for various periods of time.](image)

The distributions of $L_0$ illustrated in figure 4.19 show a similar non-parametric distribution of measurements for the control in-vitro model, which fast becomes a skewed distribution centred around a low $L_0$ from week 2 onwards. There can also be observed a small peak of high value $L_0$ in weeks 2, 4 and 8.
Figure 4.19: Stacked histograms of contour length (bin size 10 nm) of in-vitro collagen models glycated with glyoxal for various periods of time.
4.3.3.3  Methylglyoxal glycated in-vitro tissue models

Nanoindentation

Nanoindentation measurements of methyl glyoxal glycated in-vitro models yield an interesting trend as shown in figure 4.20. E was found to not significantly decrease immediately with glycation from the control model with $E_{\text{control}}=5.52\pm0.34$ GPa to $E_{\text{week1}}=5.99\pm0.30$ GPa ($p=0.014$ – not significant) and $E_{\text{week2}}=5.99\pm0.30$ GPa ($p=0.016$). However, from week 4, E was found to significantly decrease to $E_{\text{week4}}=4.46\pm0.22$ GPa ($p<0.001$). Interestingly, from week 8, E was found to be higher than the control model with $E_{\text{week8}}=5.93\pm0.18$ GPa ($p<0.001$) and $E_{\text{week10}}=7.53\pm0.29$ (p=0.005). This statistically significant increase in E at later weeks was not observed for other glycating agents.

The distributions of E for the various samples shown in figure 4.21 illustrate the change from a non-parametric distribution shown by the control and week 1 samples, to
distributions centred on an average for weeks 2, 4 and 8. The distribution reverts back to
become similar to the control at 10 weeks of incubation.

Figure 4.21: Stacked histograms of contour length (bin size 10 nm) of in-vitro
collagen models glycated with methylglyoxal for various periods of time.

Figure 4.21: Stacked histograms of contour length (bin size 10 nm) of in-vitro
collagen models glycated with methylglyoxal for various periods of time.
Contour length measurements of in-vitro models glycated using methylglyoxal illustrated in figure 4.22 show an immediate, statistically significant reduction in median $L_0$ after 2 weeks of glycation from $L_{0,\text{control}}=107.68\pm5.83$ nm to $L_{0,\text{week2}}=33.65\pm2.81$ nm ($p<0.001$) and subsequent samples with $L_{0,\text{week4}}=39.46\pm3.69$ nm ($p<0.001$), $L_{0,\text{week6}}=46.01\pm2.41$ nm ($p<0.001$), $L_{0,\text{week8}}=62.52\pm7.02$ nm ($p<0.001$), $L_{0,\text{week10}}=58.23\pm10.95$ nm ($p<0.001$).

The reduction in $L_0$ is sustained until week 8. Between week 6 and 8 there is a statistically significant increase in $L_0$ ($p=0.003$), however as described above, $L_{0,\text{week8}}$ and $L_{0,\text{week10}}$ remain lower than the control model.

![Figure 4.22: Boxplots showing contour length measurements of in-vitro collagen models glycated with methylglyoxal for various periods of time.](image)

The distributions of $L_0$ for methylglyoxal glycated samples shown in figure 4.23 illustrate a fast reduction in $L_0$ after 2 weeks of glycation, with a less clear parametric distribution observed in later weeks.
Figure 4.23: Stacked histograms of contour length (bin size 10 nm) of in-vitro collagen models glycated with methylglyoxal for various periods of time.
4.4 Key findings

4.4.1 Nanomechanics

Statistically significant reductions in both elastic modulus and contour length were observed for in-vitro tissue models glycated with glucose, glyoxal and methylglyoxal (albeit followed by an increase with this agent) after periods of incubation.

4.4.2 Understanding the control in-vitro tissue model

Beginning analysis with the control model, the distribution of elastic modulus and contour length can be seen as highly varied, spanning a variety of values with no clear defined distribution.

The measurements of elastic modulus matched those in literature (described in the introduction chapter). In particular, the work by Wenger et al. (that influenced the nanoindentation methodology used in this study) found $E$ to be distributed between 3.75-11.5 GPa, with no particular distribution described. This study found a median $E_{\text{control}}=5.52\pm0.34$ GPa, which is in line with Wenger’s work. It is important to note that Wenger measured the stiffness of collagen fibrils sourced from rat tail tendon which would be subject to a variety of covalent cross-links.

The variance in stiffness of fibrils from the control model is due to the nature of the cross-linking. Without systematic covalent cross-linking, the stiffness of fibrils will vary greatly as the extent of bonding between molecules is not standardised. The primary bonding between collagen molecules in a control model is hydrogen bonding, which varies greatly in strength and type as described in the previous chapters.
4.4.3 Hydrogen bonding revisited

We can take into consideration the 3 main types of hydrogen bond based cross-linking when exploring structural stability of a collagen fibril – intra-chain, inter-chain and intermolecular. α and γ bridges provide intra-chain hydrogen bonding, stabilising the backbone of collagen alpha chains. β and δ bridges provide inter-chain hydrogen bonding, stabilising the collagen triple helix. While they are not at a scale where they directly hold fibrillar structure intact (they are not intermolecular hydrogen bonds), they are more fundamental as they impact sub-quaternary collagen structure. Instability of the collagen backbone or the triple helix would result in a cascading effect, impacting the morphology of fibrils. Considering the lack of Lysyl oxidase mediated covalent cross-links that stabilise triple helical structure in-vivo, any disruption to α, β, δ and γ bridges would be devastating to large scale structure. The most common water bridge (δ2) involves 2 mediating water molecules and are reported to be highly stable. Intermolecular ω bridges on the other hand are less stable, with any number of water molecules mediating the bridge. They commonly involve 2 or 3 water molecules to bridge collagen molecules and provide stability for wider fibrillar structure (Bella, Brodsky et al. 1995).

Water bridges are highly transitional and dynamic, forming and breaking sometimes within very short periods of time (Ravikumar and Hwang 2008). When exposed to a highly polar environment in an aqueous environment (e.g. glycation solution), it is possible for intermediary water molecules to become interchangeable. This means that in an aqueous environment at physiological temperatures, it is possible for water mediated cross-links that aid stability of fibrillar structure to be highly transient.
4.4.4 The effect of hydrogen bonding

This dynamic nature manifests in hydrogen bond cross-linking being in a state of flux during incubation. If we consider that fibrillar structure of a control tissue model is highly dependent on this cross-linking, it is clear that the elastic modulus of fibrils will have a large variance.

Contour length measurement of the control tissue model were found to be lower than reported in literature, with a median $L_{0,\text{control}}=107.68\pm5.83$ nm. This compared to the study by Bozec et al. which, using a similar methodology found contour length to be $202\pm5$ nm. Bozec's work was focussed on individual molecules which were not affected by intermolecular hydrogen bonding to the same extent as this study. Whereas Bozec extracted individual molecules from a mica substrate, this study extracted individual molecules from fibrils. The collagen fibril provides a richer source of intermolecular hydrogen bonds and so increases the binding of the extracted collagen molecule to its fibril substrate. The tighter a molecule is bound to a substrate, the smaller the contour length (elaborated further in a following section). The large variance in contour length observed is also indicative of the transience of hydrogen bonding, with molecules not being homogenously bound across the sample.

4.4.5 Decreasing elastic modulus shows a reduction in fibril density

The decrease in elastic modulus observed across the samples is contrary to what one may expect when investigating increased cross-linking. The notion that increased cross-linking results in increased stiffness must be reconsidered to understand the interaction with fibrils brought about by nanoindentation. Indenting a fibril radially leads to a measurement of the radial stiffness and thus, indirectly the density of the fibril. This
means the decrease in elastic modulus observed in the latter weeks of glycation for glucose, glyoxal and methylglyoxal (excluding week 10) can be attributed to a decrease in the density of fibrils measured. This decrease in density occurs with increased glycation time and is an interesting feature at the fibrillar scale of AGE cross-linked models.

It may be intuitive to believe that increased cross-linking would result in increased density within a fibril as cross-links pull together neighbouring molecules. However, the results from nanoindentation show a contrasting effect – a reduction in fibril density with increased cross-linking. The results of single molecule force spectroscopy (SMFS) indicate that cross-linking is occurring with increased glycation time due to the increasing affinity of collagen to bulk fibril.

4.4.6 Decreasing contour length shows a reduction in collagen molecule affinity

The decrease in contour length exhibited by all three glycated tissue models is indicative of the increasing collagen affinity with glycation. The length of molecule extracted from the fibril is related to how closely bound it is to the fibril substrate. The longer length means more of the molecule is being pulled.

The binding between the AFM tip and the collagen molecule are primarily weak interaction forces, Van-der Waals interactions with a strength of 0.4-4.0 kJ/mol (Huheey, Keiter et al. 2006). This is significantly weaker than H-bonding with typical bond strength of 12-30 kJ/mol (Huheey, Keiter et al. 2006). And is the primary bond formed between collagen molecules in an uncross-linked, control tissue model. The contour length reflects the extent to which a collagen molecule (or several molecules bound together through hydrogen bonds) is pulled from the fibril before an unbinding event is observed. The
unbinding event occurs due to the molecule detaching from the AFM tip, at which point it returns to the bulk fibril due to its affinity brought about by hydrogen bonding (in the case of an uncross-linked tissue model). For a glycated tissue model, the affinity of collagen molecules to the bulk fibril is increased due to a series of covalent AGE cross-links bonding molecules together.

AGE cross-links are orders of magnitude stronger than hydrogen bonding, with a C-N bond strength of 293 kJ/mol (Huheey, Keiter et al. 2006) (glucosepane forms through amine groups of arginine and lysine). The accumulation of AGE cross-links increases the likelihood of glycation sites being actively bound by AGE cross-links. This means as a collagen molecule is extracted from a fibril, the likelihood that a cross-link keeps it attached to a neighbouring molecule increases. A molecular dynamic study by Collier et al. has identified 6 energetically favourable, potential binding sites for glucosepane along a collagen molecule as shown in figure 4.24 (Collier, Nash et al. 2015). For a collagen molecule saturated by glucosepane cross-links, there will be up to 6 covalent cross-links binding it to neighbouring collagen molecules.

The range of $L_0$ measured can be attributed to the length of collagen molecule extracted prior to reaching an AGE cross-link, holding it to the bulk fibril. When this point is reached, the molecule unbinds from the tip as shown in figure 4.25. The more the sample is cross-linked, the higher the likelihood that an AGE cross-link is reached and so the
lower the $L_0$. Although figure 4.24 does not provide an exact spatial understanding of the length of collagen molecule that can be extracted before reaching a glucosepane cross-link, it does illustrate the range of spatial positions of potential cross-linking sites. Sites 7, 13 and 17 in figure 2.26 are of considerable distance away from the N and C termini of the molecule, which considering its length of 297 nm (2005), provides a range of values for $L_0$ before an AGE cross-link is reached.

![Diagram](image)

Figure 4.25: Diagrammatic representation of an unbinding event. A collagen molecule bound to neighbouring molecules by hydrogen bonds (water bridges) and AGE cross-links is extracted by an AFM tip.

4.4.7 Differences in glycation properties across the different models

The nanomechanical behaviours of the samples were not homogenous, with differences in the duration for reduction as well as extent. It is clear that in-vitro tissue models glycated with glucose require a longer duration of time before changes to nanomechanical properties are observed – for both reductions in elastic modulus and contour length. The pattern of decrease is of special interest.

Nanoindentation of collagen fibrils from glucose glycated models interestingly show an initial reduction in elastic modulus after 5 weeks of incubation followed by an increase at
weeks 6 and 7 and then an eventual decrease in elastic modulus at weeks 8, 9 and 10. The reduction of elastic modulus indicates the density a decrease of density within collagen fibrils with the accumulation of AGE cross-linking. This decrease in density is sustained over the course of latter weeks and is foreshadowed by an initial, brief increase in density. These changes at later weeks of incubation with glucose can be attributed to the accumulation of AGE cross-links rather than other potential degrading changes to fibrillar properties through incubation as discussed earlier. This is due to an initial imaging scan to identify each fibril to be indented, prior to indentation. The selection of clearly intact fibrillar structure negates the possibility that changes observed in nanomechanical properties are due to fibrillar structure dissociation.

A somewhat similar pattern is observed in changes of the contour length measurements. An initial increase is observed at weeks 4 and 6, followed by a decrease in weeks 8 and 10. This time, the collagen affinity is found to decrease (indicated by an increase in $L_0$) followed by an eventual increase in collagen affinity (indicated by a decrease in $L_0$). The implication is that as glycation time increases, there is an initial relaxation of collagen molecule affinity caused by the AGE cross-links between weeks 4-6, followed by a tightening of affinity from week 8.

Glyoxal glycated models displayed an almost immediate and sustained decrease in elastic modulus, indicating a sustained decrease in fibril density with the accumulation of AGE cross-links. This is supplemented by a sustained reduction in collagen molecule affinity with contour length decreasing over the 10 week glycation period. Methylglyoxal glycated models however, show a decrease in the density of collagen fibrils followed by an increase in the later weeks of glycation. A similar, unexpected pattern is observed in contour length measurements, indicating the loss of collagen molecule affinity over time.
The fact that the pattern is observed in both experiments implies that methylglyoxal based AGES are declining over time (the impact of which is discussed in the following section).

4.4.8 The importance of AGE stability

The susceptibility of methylglyoxal adducts to decrease in concentration over time has been reported in a study by Ahmed et al (Ahmed, Thornalley et al. 2003) which found that the increased concentration of the most common methylglyoxal adduct, MG-H1 (methylglyoxal hydroimidazolone-1) with age required a sustained increase in the concentration of methylglyoxal over time. This is due to the requirement to reach a balance between the formation and degradation of these AGES. The most common methylglyoxal adducts MG-H1 has a half-life of approximately 11.7±2.7 days (Ahmed, Argirov et al. 2002, Thornalley, Battah et al. 2003); far lower than the half-life of the most common glyoxal adduct G-H1, which has a half-life of approximately 69±16 days.

Interestingly, a study investigating the stability of AGES by measuring the time taken for a 5% change in assay from initial value (t$_{95\%}$) found MG-H1 had t$_{95\%}$=15±5 days and G-H1 had t$_{95\%}$=41±7 days (Ahmed, Argirov et al. 2002).

More importantly however, the study found that MOLD had a t$_{95\%}$ of 1.1±0.4 days and GOLD had a t$_{95\%}$ of 6.3±0.1 days (Ahmed, Argirov et al. 2002). It is clear that methylglyoxal adducts are less stable than glyoxal adducts, with this relevant to bis(Lysyl) imidazolium cross-links MOLD and GOLD too. In the context of this study, this explains the apparent loss in the impact of glycation for the methylglyoxal glycated model after 6 weeks of incubation.

Methylglyoxal derived adducts, including MOLD are less stable than glyoxal derived adducts. A continually increasing concentration of MOLD relies on a sustained increase
in the concentration of methylglyoxal. For the in-vitro glycated models, methylglyoxal concentration (100 mM) was indeed lower than glyoxal concentration (800 mM) in their respective glycation reagents. Both glycation reagents far exceeded physiological concentration of glycating agent and were sufficient to cross-link the in-vitro models. However it is thought that the concentration of methylglyoxal was not sufficient to continuously increase the number of methylglyoxal mediated adducts by replenishing completely adducts formed and broken after 6 weeks of incubation. This describes the loss of the functional properties induced by cross-linking at this time for both nanoindentation and SMFS experiments. The number of AGEs formed for the first 6 weeks were sufficient to replenish any unstable AGEs while increasing AGE formation. After this time, it is possible that the reagent was not sufficient to increase the number of MOLD cross-links and MG-H adducts (among other AGEs) and so the impact of such adducts became less pronounced. A way of testing this theory is to continuously add glycating agent to the in-vitro tissue model, increasing the concentration over the course of the incubation period, providing a continuously rich source of methylglyoxal molecules for glycation.

4.4.9 Proposed mechanism: AGEs provide an additional form of collagen-hydration interaction, increasing fibril hydration and decreasing fibril density

In section 4.4.4 we explored the decrease in fibril stiffness as a function of glycation time. It is proposed that this is due to a decrease in fibril density, brought about by increased concentration of AGE products formed over the glycation period. The corresponding decrease in contour length shows the existence of cross-links, increasing the binding affinity of collagen molecules.
To explore why the density of fibril decreases with cross-linking, we must consider the chemical structure of AGEs formed during the glycation period. All AGE cross-links contain polar groups, whether they are amines, carbonyls or hydroxyl groups. If we consider the chemical structure of glucosepane, the complex contains a number of highly polar groups as shown in figure 4.26 (Sell, Biemel et al. 2005), specifically two hydroxyl groups and an imidazole ring.

Figure 4.26: The polar groups (hydroxyls and imidazole) found in glucosepane are circled in blue.

The presence of hydrophilic groups suggests the glucosepane complex exhibits high water affinity, however the energetic favourability of glucosepane-water interaction is of vital importance for this to occur. A collaborator at the Department of Chemistry, UCL, was asked to model the interaction of water with glucosepane (Nash, Sassmannshausen et al. 2017). Building upon the hypothesis from this study, the author found the formation of hydrogen bonds to be highly energetically favourable for both hydroxyl groups and both nitrogen atoms at the imidazole site – with the imidazole site exhibiting higher water affinity than the hydroxyl groups. What this shows is the potential for glucosepane to provide increased hydration properties to collagen. As glucosepane accumulates within a collagen fibril, the water retention properties of the fibril increase. This leads to increased fibril hydration with increased glycation. In addition to this point, if we
consider further AGES, such as GOLD, MOLD, G-H and MG-H, we can see the presence of imidazole sites as shown in figure 4.27.

Figure 4.27: AGES (glyoxal-hydroimidazolone, methylglyoxal hydroimidazolone, glyoxal Lysyl dimer and methylglyoxal Lysyl dimer) with imidazole sites, providing potential hydrogen bonding sites (Thornalley, Langborg et al. 1999).
This provides the potential for AGES (cross-links and other adducts) to allow increased water-interaction to collagen molecules and so increased hydration glycated collagen fibrils. AGEs add to a collagen molecule’s ability to attract hydration shells due to hydrophilic groups binding interstitial waters. This presents a new mode of water interaction, adding to the 3 currently known modes, which are illustrated in Figure 4.28(i). The green water molecules represent water covalently bonded to triple helix, hydrogen bond based water bridges are represented by the purple water molecules and interstitial waters represented in blue. The added AGE based hydration is illustrated in Figure 4.28(ii) by the blue water molecules.

With this in mind, nanoindentation results are affected by water flow within collagen fibril, illustrated by the light blue water molecules. As AGEs accumulate, more water molecules may be bound within the fibril as shown in Figure 4.28(ii). As water binds within the fibril, its density decreases, leading to a decrease in the radial stiffness of the fibril. The indentation process rather than compressing the collagen fibril directly begins probing the properties of water flow within the fibril, as in Figure 4.28(iii), before the indentation of the fibril occurs. The overall indentation therefore is constituted by both a collagen fibril compression component and a water flow component, thus reducing the transverse Young's Modulus. This hypothesis resides on an affinity between interstitial water present in fibrils and AGE mediated crosslinks such as Glucosepane.
Figure 4.28: Illustrations for the three known modes of water interaction with collagen molecules; i) covalently bonded to the molecule (a, green), water-bridges between molecules (b, purple) and free flowing water (c, blue). ii) The proposed mode of water interaction: water retention caused by Glucosepane accumulation (d). iii) Proposed mechanism of water flow within collagen fibril caused by nanoindentation.
4.4.10 Wider studies of fibril hydration in literature

Increased fibril hydration has been shown to affect the width of collagen fibrils by a number of studies (Grant, Brockwell et al. 2009). Importantly, swelling of fibrils has been shown using AFM, by measurement of fibril height (Grant, Brockwell et al. 2008, Svensson, Hassenkam et al. 2010). This can be corroborated by studies investigating the impact of hydration on collagen fibril stiffness. The most relevant of which is a nanoindentation study by Grant et al. (Grant, Brockwell et al. 2008) which found a decrease of elastic modulus by 3 orders of magnitude comparing hydrated and dehydrated (air-dried) collagen fibrils. The measurements carried out on bovine Achilles tendon samples show the extent of decrease of radial stiffness brought about by hydration. Theoretical investigations also support the finding that swelling causes a decrease in radial stiffness of fibrils (Morin, Hellmich et al. 2013).

The relationship between cross-links, increased hydration has been observed before in riboflavin mediated cross-linked in-vitro collagen models. A study showed the water retention properties of cross-linked collagen model being higher than that of a control model (Rich, Odlyha et al. 2014), setting a precedence for cross-link aided collagen-water interaction.

### 4.5 Conclusions

The aim of this chapter was to investigate as a function of glycation, the change in biophysical and structural properties of collagen. This was to be carried out by introducing cross-links to a purely collagen substrate in a controlled manner, before probing the various morphological and mechanical properties of the model at the nanoscale.
The first objective was to develop an in-vitro glycated tissue model to simulate the ageing process. This was achieved with glucose, glyoxal and methylglyoxal models, all of which showed signs of browning during the incubation period. The in-vitro models were structurally sound (lasting the entire incubation period) and suited to the host of characterisation techniques used in this study. There were limitations of the models produced, the main being the susceptibility to bacterial contamination. This was resisted by ensuring tissue models were kept sealed from point of production (and glycation) until the time-point for characterisation. However, this meant the glycation reagent could not be added to and so the concentration of methylglyoxal was insufficient for increased AGE concentration as describe in section 4.4.6.

The second objective was to identify changes in the in-vitro models as a function of glycation, as a way of identifying potential biomarkers of ageing. Changes in morphology were observed in all three types of glycated tissue models. These included occurrence of fibril bundling and localised alignment among fibrils. Changes in the nanomechanical properties were observed among the glycated models. This included a decrease in the radial stiffness of individual collagen fibrils, indicating a decrease in fibril density due to increased hydration. The discovery that contour length decreased with glycation showed that affinity of collagen molecules was also found to increase with glycation.

This study proposes a new water interaction mechanism related to the accumulation of AGEs. This is the first time AGE cross-links and other adducts have been shown to affect water interaction with collagen and shown to impact nanoscale properties. This needs to be explored in ex-vivo samples to test the physiological relevance of the theory.
5 Investigation of Ageing of Dermis

5.1 Introduction

After having carried out an in-vitro model, it is important to expand the study further by looking at ex-vivo tissue samples. It is vital that potential nanoscale markers of ageing identified are assessed in physiologically relevant conditions. The aim of this chapter is to identify potential markers of ageing in human skin, while assessing the existence of previously identified markers of ageing in the glycated tissue models from chapter 4. This will be achieved using the following two objectives:

1. Prepare human tissue samples for characterisation using nanoscale techniques
2. Apply the tools and techniques developed in chapter 4 to human skin – namely nanomorphological and nanomechanical analysis.

Dermis provides a useful tissue sample, being of especial interest when it comes the deleterious effects of ageing. As described in Chapter 1, dermis is 2-3 mm thick and is composed of multiple layers with different characteristics and functions. The layer under consideration in this study was the reticular dermis, with fibrillar collagen in particular being the dermal component being investigated. Presented in the following section are challenges faced in ensuring suitable characterisation of the sample to achieve the aim of this study.

5.2 Preparing samples for application of techniques

Samples were prepared by cryosectioning and staining as described in chapter 3. Preparing ex-vivo tissue for analysis with nanoscale techniques proved difficult due to the novel nature of characterisation. The methodology described in Chapter 3 was the
culmination of a number of pilot stages, yielding less than satisfactory results. Table 1 provides basic information about the 11 healthy volunteers who took part in this study.

<table>
<thead>
<tr>
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<tr>
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<td>79</td>
</tr>
<tr>
<td>Female</td>
<td>Caucasian</td>
<td>82</td>
</tr>
</tbody>
</table>

Table 5.1: Gender, race and age of participants in this study

5.2.1 Cryosectioning tissue

After collection of tissue from volunteers, the cryosectioning technique was used to prepare tissue samples for characterisation. While cryosectioning of dermis is a standard process for histology, this is also required to be adapted for AFM analysis due to the highly flat surface required for imaging. It was hypothesised that thin tissue samples would provide ideal characterisation conditions. However, there was a minimum limit of section thickness after which sample quality deteriorated. Presented in figure 5.1 is an example of a dermis sample from a 26 year old volunteer. Figure 5.1(a) has been stained in
Haemotoxin and Eosin (H&E) and shows clearly the epidermis and dermis, with cells stained as blue dots. Figure 5.1(b) shows the section stained in Pico-sirius red (PR), with collagen fibres stained deep red. The section is 8 µm thick and provides a clear illustration of the structure of the tissue.

Comparing this to the section showed in figure 5.2, which is of a 26 year old volunteer, stained in PR, but sectioned to a thickness of 5 µm, we can see a very clear loss in tissue density. Cryosectioning the tissue thinner than 8 µm seems to provide unsatisfactory sample quality, with a significant loss of tissue content. The skin samples were sectioned to a thickness of 8 µm for the study.
Cryosectioning in effect takes a cross-section of a sample, with the orientation of fibres being of great importance. Skin contains fibres oriented in many directions, as collagen sheets have different orientations. Cryosectioning may slice tissue across these sheets rather than along - There were several instances of fibres being sectioned across their diameter rather than along their long axis. These planes of collagen presented regions of tissue which were not points of interest though interesting artefacts of sample preparation. The identification of these sites emphasise the importance of imaging prior to further nanomechanical analysis as it’s important to be certain fibrillar collagen is being probed. These orthogonal planes were observed using both SEM and AFM as shown in figure 5.3(a) and (b) respectively, with planes of fibrils sectioned across their diameter being circled in red.

![Figure 5.3: Planes of collagen sheets shown by SEM and AFM from a 28 year old (a) and 32 year old (b).](image)

5.2.2 Characterising skin

As described in chapter 1, skin is a complex and multi-layered organ with the dermis itself containing two sections, papillary and reticular. Figure 5.4(a) shows the epidermis (i), the papillary dermis (ii) and the reticular dermis (iii). The stratum corneum can be shown by the light pink layer above the epidermis. The epidermis, a cell rich region is attached
to the basement membrane found at the dermal-epidermal junction, which is attached to the papillary dermis, a collagen rich region. The dermal-epidermal junction is shown by figure 5.4(b). The difference in microstructure between the two layers can also be seen in 5.4(b)(i) and (ii), with cells in the type-III collagen rich papillary dermis being attached to the basement membrane by type-VII collagen anchoring fibrils.

5.2.3 Non collagenous components of the dermis

Not only is skin a multi-layered tissue, it also contains numerous non collagenous components which must be avoided during nanomechanical analysis. Cells such as fibroblasts (*) make an important component of the papillary dermis as shown in figure 5.5(a) and (b). Whilst it is clear from figure 5.5(a) that the epidermis has the highest cell density, the papillary dermis also has a high cell density as shown in figure 5.5(b).
The reticular dermis provides a high source of collagen rich areas, with a lower cell density than the papillary dermis, however this is still interspersed with cells as shown in figure 5.6. In order to avoid probing into the nanomechanical properties of non-collagenous components of the dermis, it is important that analysis is focused exclusively on the reticular dermis. The presence of cells in this section emphasises the need to image a region first prior to nanomechanical analysis. This can be performed using an inverted microscope upon which is mounted the AFM used in this study.

Figure 5.5: Histology of 73 year old skin section illustrating the papillary dermis at 10x (a) and 20x (b) magnification. * displays the presence of fibroblasts.

Figure 5.6: Histology of an H&E stained 32 year old skin section, illustrating the reticular dermis at 10x magnification.
Although the reticular dermis is a rich source of collagen, it also contains the fibrous protein elastin. Excessive non collagenous, fibrous components such as elastin can result in decreased collagen staining as shown in figure 5.7. This sample displays characteristics of increased elastin content. The lack of high collagen content in this section emphasises the need to image for fibrillar collagen prior to nanomechanical analysis.

Figure 5.7: Histology of 28 year old PR stained skin section, showing low collagen content in the reticular dermis.

5.2.4 Microscale to nanoscale analysis with electron microscopy – the pitfalls

Electron microscopy was a powerful tool for imaging dermis at both microscale and nanoscale resolutions as demonstrated in figure 5.3(a) and figure 5.4(b) respectively. However there were significant disadvantages and flaws in using this method to identify potential nanomorphological markers and subsequently nanomechanical markers.

Firstly, the prevalence of features in the collagen matrix, resembling “cracks” were observed commonly in electron microscopy images. Not observed in AFM imaging, it is thought that these are desiccation cracks, caused by the vacuum environment that samples were placed in. These present imaging artefacts which do not represent morphology at both the microscale and nanoscale as shown in figure 5.8(a) and 5.8(b),
respectively. While the desiccation cracks allowed imaging of fibrils deep within the matrix, exemplified in figure 5.8(b), they are not physiologically relevant and introduce artefacts in the morphological structure of the collagen matrix.

Secondly, the requirement of sample fixing and a conductive surface coating also mean nanomechanics cannot be performed on samples prepared for SEM. Investigation of nanomechanical properties must be performed on samples closely resembling physiological conditions, without introducing the mechanical response from a gold surface coating.

Figure 5.8: SEM of skin section from a 23 year old, at low resolution (a) and high resolution (b).
5.3 Results

5.3.1 Histology

Presented in figure 5.9 are representative optical images obtained from stained histological sections of young: 28-year-old (a & b) and old: 82-year-old, volunteers (c & d).

Figure 5.9: Histology sections stained in Haemotoxin and Eosin (a), (c) and Pico Sirius Red (b), (d) for a 28-year-old and an 82-year-old volunteer, respectively. * displays thicker epidermis, ** displays collagen stratification.
Distinct differences in dermis ultrastructure were observed using a standard light microscope with a 20x objective. Images (a) and (c) obtained from the H&E stained samples showed significant variation in the epidermis layer thickness (darker pink layer) in the older volunteer whereas the epidermis thickness remained more homogenous in the younger volunteer (*). However the biggest structural difference was observed in the Pico Sirius Red stained sections. Both young and old volunteer sections displayed significant staining suggesting as expected a high collagen content in the dermis. However, the overall ultrastructure of the collagen scaffold within the dermis varied considerably; older dermis appeared to be porous with large gaps intercalated between collagen stratified layers (**) while younger dermis was denser, though discrete large gaps were found within the section.

5.3.2 Microscale morphology

As shown by various images presented in section 5.2, SEM provided effective imaging of the microstructure of the reticular collagen matrix. Figures 5.10(a), (b), (c) and (d) provide representative images of dermal tissue from young volunteers. The images show a dense collagen matrix at the microscale, with fibres and bundles of fibres difficult to distinguish within the surface (*). Individual collagen fibrils can only be seen at the tissue section boundaries, shown in figure 5.10(b) or within desiccation cracks (**), shown in figure 5.10(d). Overall, the porosity within the tissue at the microscale is primarily due to desiccation cracks, an artefact of the imaging rather than a feature of the sample.
The microstructure of tissue from older volunteers present a significantly different series of features as shown by figure 5.11(a), (b), (c) and (d), representative images of old tissue. Porosity is apparent throughout the collagen matrix at low magnification (*). Unlike the younger tissue in which porosity was predominantly due to desiccation cracks, in this case, it is a feature of the tissue itself. The porosity can be seen to be due to differentiated bundles of fibres in the reticular dermal matrix (**). Fibrillar structure can be seen as fragmented with no overall cohesion, porous with a cluster of interstitial gaps appearing in between fibril bundles. Interestingly figure 5.11(b) shows a tissue sample from a middle aged volunteer (60) – presenting symptoms of both young and old tissue; low porosity coupled with differentiation of fibres within the collagen matrix.

Figure 5.10: Low magnification SEM images of young tissue; aged 26 (a), 28 (b), 23 (c) and 30 (d). * marks indistinguishable fibril bundles, ** marks desiccation cracks
5.3.3 Nanoscale morphology

Interestingly, high resolution SEM of dermal matrix from young volunteers did not provide effective imaging of ultrastructure as shown in figure 5.12. It is difficult to distinguish fibrillar structure in the dermal matrix (*), shown by figure 5.12(a), with fibrils being observed only at desiccation cracks (**), shown by figure 5.12(b).

Figure 5.11: Low magnification SEM images of old tissue; aged 73 (a), 60 (b), 73 (c) and 82 (d). * marks porosity, ** marks fibril bundles

Figure 5.12: High magnification SEM images of young tissue; aged 26 (a) and 28 (b). * marks indistinguishable matrix structure, ** marks fibril presence
In contrast to this, heterogeneous nanoscale structure of old tissue samples could easily be observed using SEM, illustrated by representative images of older tissue samples in figure 5.13. Bundles of fibrils can be observed in figures 5.13(a), (c) and (d). Porosity also seems to extend to the nanoscale as illustrated by (b), a result of interstitial gaps between fibrils and bundles of fibrils (*). The alignment of fibrils and fibril bundles seems to be randomly oriented, shown by (a) and (b), and multiple fibril bundles join together to create larger bundles (**) as shown in (c). An interesting “swirling” orientation can be observed in (d), with fibril bundles creating wave-like patterns (***)

Figure 5.13: High magnification SEM images of old tissue; aged 73 (a, b), 82 (c, d). * marks interstitial gaps, ** marks fibril bundles, *** marks wave-like fibrillar arrangement
In order to ensure these nanoscale features are characteristics of older tissue, we must compare the images to high resolution AFM imaging of younger tissue, provided in figure 5.14(a) and (b).

Fibrils in figure 5.14(a) show highly contrasted D-banding periodicity (*), the fingerprint for collagen quaternary structure. The fibrils show a high degree of alignment, with dense packing (**). Figure 5.14(b) shows the intersection of two dense collagen planes, showing that dense, banded collagen is also present deep within the dermal matrix (**). Overall, the ultrastructure of the collagen scaffold from younger volunteers can be summarised as a dense, compact scaffold with highly contrasted D-banding periodicity.

Changes in quaternary structure of dermal collagen with ageing are exemplified in Figure 5.14(c) and (d), which show collagen fibrils in a degraded state without characteristic D-banding periodicity. The fibrils display significant signs of swelling (****), a possible result of fibril bundling. Overall, the ultrastructure of the collagen scaffold from older volunteers can be summarised as a porous, fragmented scaffold with loss of D-banding periodicity.
5.3.4 Fibril swelling – an initial study

To assess the assumption, based on observations, that fibrils from the aged tissue exhibited signs of swelling, a preliminary measurement of fibril diameter was performed using AFM topographical analysis, illustrated in figure 5.15. Line profile measurements on fibrils from old tissue provided an average fibril width and height of 412.7±91.0 nm and 229.2±52.3 nm respectively (N=30). This was higher than measurements on fibrils from young tissue which provided average fibril width and height of 114.5±13.6 nm and
78.5±9.7 nm respectively, with two tailed t-tests showing a statistically significant difference (p<0.001). It is important to note that this method does not provide accurate fibril diameter measurements for a variety of reasons, including fibrils flattening onto the substrate, leading to a collapsed, elliptical shape – alluded to by the difference in height and width provided. Another reason is a reliance on the sharpness of the AFM tip for accurate width measurement.

Figure 5.15: Profile line across a fibril in an AFM topography image

5.3.5 Nanoindentation

Systematic nanoindentation measurements were taken directly on the histological sections obtained from the volunteers. From the series of localised force-distance curves collected exclusively on collagen fibrils, the elastic moduli of fibrils in the radial direction were calculated using the Oliver-Pharr model as described in chapters 2 and 3. The measurements for tissue from young volunteers are presented diagrammatically in Figure 5.16. Unless stated otherwise, samples were air-dried and analysed in a dehydrated state.
5.3.5.1 Young volunteer tissue samples

The distributions presented by the histograms display large variation in stiffness across the young volunteer samples. They however centre on mean values ($E_{\text{age}}$) which are: $E_{21}=5.04\pm0.64$ GPa, $E_{23}=6.82\pm0.32$ GPa, $E_{26}=5.60\pm0.59$ GPa, $E_{28}=6.49\pm1.01$ GPa, $E_{30}=6.33\pm1.08$ GPa and $E_{32}=2.53\pm1.33$ GPa. Interestingly, nanoindentation of fibrils from the 32 year old exhibited a smaller elastic modulus compared to the other samples. This difference was also reflected in its distribution, shown in figure 5.16.

![Figure 5.16: Histograms showing the distribution of elastic modulus for dermal fibrils from young volunteers.](image)

5.3.5.2 Old volunteer tissue samples

When it comes to nanoindentation of fibrils from tissue of old volunteers, a change can be observed in the distribution of elastic modulus, shown in figure 5.17. The distributions
have a smaller variance with a more pronounced normal distribution (excluding the 68 year old tissue sample). The mean elastic moduli for the samples in this case are; $E_{60}=3.64\pm0.52$ GPa, $E_{68}=2.61\pm0.99$ GPa. $E_{73}=2.96\pm0.44$ GPa, $E_{79}=2.95\pm0.33$ GPa and $E_{82}=3.01\pm0.38$ GPa. Interestingly this suggests that the elastic modulus of fibrils from older tissue present lower values than for younger patients.

![Figure 5.17: Histograms showing the distribution of elastic modulus for dermal fibrils from old volunteers.](image)

5.3.5.3 Young vs old volunteer tissue samples

Comparison of nanoindentation results from both sets of volunteers shows a distinct difference in the distributions of the elastic modulus of collagen – shown by histograms in figure 5.18. Young tissue (highlighted red) have broad distributions of values, including
a large proportion of fibrils with high stiffness. Old tissue (highlighted black) however, show narrower distributions, centred on lower mean elastic modulus.

Figure 5.18: Histograms showing the distribution of elastic modulus for dermal fibrils from young (red) and old (black) volunteers.
The pattern of older tissue providing fibrils with lower radial stiffness than those provided by young tissue is further exemplified by boxplots in figure 5.19.

![Figure 5.19: Boxplots showing the distribution of elastic modulus for dermal fibrils from young and old volunteers.](image)

Interestingly tissue samples from the 32 year old and 68 year old are anomalous when comparing to their respective cohorts (young and old). The 32 year old presented a narrow distribution of $E$ with a lower mean $E$ compared to the remaining cohort of young tissue samples. Similarly, the 68 year old presented a wide distribution of $E$ in comparison to the remaining old tissue samples. The differences were not statistically significant compared to their respective cohorts ($p>0.001$, two tailed t-test) however, thus we may incorporate these results in our analysis as random, unknown variation.

### 5.3.5.4 The impact of Ethnicity

There may be differences in properties of the dermis due to the ethnicity of the volunteer (table 5.1). It is commonly accepted that melanin content varies with ethnicity, with melanosome density, packing and degradation dependent on ethnicity (Kaidbey, Agin et
al. 1979, Iozumi, Hoganson et al. 1993, Brenner and Hearing 2008). The thickness of the dermis has also been found to change depending on ethnicity of skin too (Montagna 2012). The 32 and 68 year old volunteers were of Bengali and Afro-Caribbean ethnicity respectively, however the 23 and 30 year old volunteers were of Indian and Afro-Caribbean ethnicity too, and so would have produced similar features in distribution of E measured. Thus we may discount ethnicity as a further variable for measuring properties of ageing using nanoindentation.

5.3.5.5 Young vs old tissue: A statistical approach

Pooling the data together into two sets, young (measurements from tissue 32 years and younger) and old (measurements from tissue 60 years and older) allows analysis of the two populations to determine whether the decrease in E observed with age, is statistically significant. Comparisons within a population was carried out using ANOVA, while comparison between populations was carried out using Student’s t-test as described in section 3.20.

Immediately from figure 5.20(a) and (b), a distinct difference in the distribution of fibrils from old (a) and young (b) volunteers can be seen. While both present a skewed normal distribution, the skew for fibrils from older volunteers is more pronounced towards a lower E. The mean elastic modulus for all young volunteers was $E_y= (8.17 \pm 0.42)$ GPa, $N_{fibrils} = 775$ and the mean elastic modulus for all older volunteers was $E_o= (4.42 \pm 0.26)$ GPa, $N_{fibrils} = 597$ and showed a statistically significant (two-tailed Student’s t-test) reduction in elastic modulus with age ($p<0.001$). The variance of the elastic modulus was also found to decrease for older volunteers ($\sigma_y^2 = 138.26$ GPa, $\sigma_o^2 = 40.29$ GPa). There were
no significant differences (using one-way ANOVA) found between volunteers within each cohort, young or old (p>0.001).

Figure 5.19: Histograms showing the distribution of elastic modulus for dermal fibrils from young and old volunteers in a dehydrated environment

5.3.5.6  **The effect of sample hydration**

As discussed in chapter 4, the measurement of collagen mechanical properties by AFM (Ghoreishi 2000) has been shown to change depending on the level of fibrillar hydration
(Grant, Brockwell et al. 2008, Grant, Brockwell et al. 2009). To evaluate the impact of hydration on the mechanical properties of collagen, two sets of measurements were carried out on the same sections, first in a dehydrated environment followed by a repeat of the same measurements after 10 minutes rehydration in PBS.

Upon viewing the distribution of elastic modulus shown by the histograms in figure 5.20(a) and (b), it is clear that the difference in fibril elastic modulus between old and young tissue observed in figure 5.19 is no longer present in hydrated tissue.

The variation in fibrillar elastic modulus independent of age followed trends in the literature, which found a significant increase in fibril modulus upon dehydration (Pasquali-Ronchetti and Baccarani-Contri 1997).
In the case of hydrated fibrils, the mean hydrated fibril modulus of the old volunteer $E_{o-hyd} = (0.89\pm0.05) \text{ MPa}$ $N_{fibrils} =64$, displayed in Figure 5.20(a) and for the young volunteer was $E_{y-hyd} = (1.24\pm0.09) \text{ MPa}$ $N_{fibrils} =63$, as shown in Figure 5.20(b). Performing two tailed t-tests showed there to be no significant difference ($p>0.001$) between elastic modulus measurements from the 82 year old volunteer and the 21 year old volunteer.

Figure 5.20: Histograms showing the distribution of elastic modulus for dermal fibrils from a 21 year old and 82 year old volunteers in a hydrated environment
5.4 Key findings

5.4.1 Morphological markers

5.4.1.1 Microscale

Microscale morphological markers of ageing were identified using light microscopy and SEM. Histology sections displayed porosity and fragmentation of the dermis. This has been reported previously in a study involving volunteers (aged 2-85 years with 121 skin samples) (Cole, Chan et al. 1996). Bonta et al. reported changes in the morphology of collagen fibres in volunteers over the age of 50, including fragmentation and disorganisation of thick collagen fibres, lysis of thin fibres and an overall decrease in matrix density.

5.4.1.2 Nanoscale

More importantly, nanoscale morphological markers were identified by observing changes in quaternary structure with SEM and AFM imaging. This study showed an age related decrease in collagen matrix density and loss of homogeneity of fibres. This supports earlier reports of changes at the macroscale (Kannus and Jozsa 1991) as well as a previous AFM study reporting nanoscale morphological changes with age. In Fenske and Lober's study, collagen fibrils in young skin (age 21) were also found to be tightly packed and aligned in comparison to sparse, fragmented and disorganised fibrils in old skin (age 55) (Promislow 1994).

It is also suggested that bundling of fibrils occurs with ageing. Observational studies showed such features in aged tissue and they were not observed in young tissue. There was also a swelling of individual fibrils in more aged tissue, supported by observational assessment as well as preliminary topographical assessment of fibril line profiles. This was correlated with the loss of D-banding.
5.4.1.3 **Morphological markers as a result of AGE accumulation**

Fibril bundling implies an increased affinity of collagen fibrils to one another. In chapter 4, the formation of intra and intermolecular AGE cross-link was explored and the effects of which hypothesised to introduce alignment, register and bundling among fibrils in an in-vitro tissue model. The bundling observed in ex-vivo dermal collagen from aged volunteers is in line with the findings of the in-vitro study. In this case, as the volunteer has aged, more AGE cross-links (predominantly glucosepane) have accumulated within the dermal collagen. These cross-links form between arginine on one collagen molecule and lysine on another – potentially in a neighbouring fibril. It may be possible for glucosepane and other AGEs to cross-link in an interfibrillar manner, thereby increasing fibril-fibril binding, leading to fibril bundling.

The observation of fibril swelling in older tissue is also due to the accumulation of AGE products. We know that the older tissue samples will have a higher concentration of AGEs accumulated onto the collagen matrix. Considering the proposed water interaction mechanism from chapter 4, fibril swelling in ex-vivo skin, may be a result of increased hydration – primarily due to AGE induced water retention. As described in section 4.4.8, hydration is known to cause fibril swelling (Grant, Brockwell et al. 2008, Grant, Brockwell et al. 2009, Svensson, Hassenkam et al. 2010).

5.4.2 **Elastic modulus of individual fibrils decreases with age**

An age-related decrease in the Young’s modulus of the transverse fibril was observed, from 8.17 GPa in young volunteers to 4.42 GPa in old volunteers (p<0.001).
In this study collagen fibrils were indented radially rather than longitudinally along the fibril axis. Thus the Young’s Modulus obtained is the transverse modulus measuring indirectly the density of the fibril as it is compressed. The age related reduction in transverse stiffness therefore relates to a change in the density of the fibril. Since all the samples were dehydrated passively in air, it is likely that any fibrillar interstitial water is no longer present. Taking this into consideration as well as the experimental data obtained, we proposed that the decrease in density of the collagen fibril with age is related to the level of water retained within the fibril.

As discussed in chapter 4, ageing increases the concentration of AGEs in dermal collagen, thereby increasing the ability of collagen fibrils to retain water. The increased fibril hydration leads to a decrease in the density of the fibril and so a decrease in its stiffness.

The age related reduction in air dried fibril stiffness is not consistent with earlier reports investigating the biomechanical properties of skin, which have overwhelmingly reported increases in stiffness (Ravelojaona, Robert et al. 2009). A number of in vitro and ex vivo studies have investigated the mechanical properties of various tissues as a function of AGE accumulation due to both ageing and diabetes (severe AGE accumulation occurs due to elevated blood glucose concentration (Robert, Moczar et al. 1974)). An age related stiffening of tissue was observed in tendon, vascular and myocardial tissue, cartilage and skin (Kuivaniemi 1985, Shapiro, Endicott et al. 1991, Knott and Bailey 1998, Langton, Griffiths et al. 2013). A stiffening of tissue was also observed from diabetic volunteers. A notable in vitro study reported an increase in stiffness of rabbit tendon incubated with glucose (Lehmann, Querings et al. 2004). Ex vivo measurements of skin auto-fluorescence have shown that the concentration of AGEs in skin increases greatly with age (Shapiro,
Endicott et al. 1991) with an age dependent stiffening observed (Ravelojaona, Robert et al. 2009) supporting earlier reports (Gunathilake 2015).

There were however, a number of ex vivo studies looking at non-AGE specific age related changes in Achilles tendon (Bailey 2001) and gastrocnemius tendon (Pageon, Zucchi et al. 2015) which reported a decrease in stiffness with age. One report investigating the Young’s modulus of dermal matrix for volunteers aged between 26-66 years (n=20) reported an increase in stiffness in the dermis for the 26-55 age group followed by a reduction in stiffness for volunteers aged in their 60s, though this was not investigated or explained in further detail (Brenner and Hearing 2008). The stiffness of hydrated samples was measured, indenting with a 5µm glass sphere mounted to a soft (k=0.02Nm⁻¹) cantilever. Thus, indentation of skin measured overall stiffness of the collagen matrix including multiple fibrils and other components of skin (elastin, hyaluronic acid and proteoglycans), and so, the macroscale mechanical properties of bulk tissue rather than those of individual fibrils as presented in this study.

5.4.3 Age dependent reduction is not apparent in hydrated sample

The measurement of collagen mechanical properties by AFM (Ghoreishi 2000) has been shown to change depending on the level of fibrillar hydration (Grant, Brockwell et al. 2008, Grant, Brockwell et al. 2009)

The lack of statistically significant difference in Young’s modulus of hydrated fibrils between young and older volunteers suggests that transverse elastic moduli for collagen in fully hydrated environments is not impacted by the age of volunteers. However, it is important to note that complete rehydration does not accurately mimic physiological conditions.
As collagen fibrils become saturated with water, it leads to increases in fibril diameter (Rich, Odlyha et al. 2014). There is little evidence that this water saturation happens in-vivo, therefore the results of the mechanical investigation of fully hydrated fibrils have to be considered carefully.

5.4.4 Proposed hypothesis - Increased hydration is an evolutionary response to loss of other hydrophilic components

The increased accumulation of AGE cross-links coincides with age dependent changes of other major components of the dermis, primarily the reduction of GAG and hyaluronic acid. The effect of GAG reduction is three-fold; firstly the loss of the hydrophilic GAG chain reduces the water storage capacity within the dermis. Secondly, decorin GAGs bind to type-1 collagen, aiding fibrillogenesis and maintaining fibril structure (Chanoki, Ishii et al. 1995, Gosline, Lillie et al. 2002). Thirdly, decorin binding has been shown to inhibit cleavage of collagen by matrix metalloproteinase (the loss of decorin GAGs may result in increased enzymatic degradation of collagen fibrils (Rich, Odlyha et al. 2014) leading indirectly to a change in the collagen matrix).

The loss of hyaluronic acid and GAG chains from proteoglycans results in a significant age related reduction of the capacity of the dermis to retain water, dramatically changing the mechanical properties and appearance of the extracellular matrix. This study suggests that the accumulation of AGE cross-links, which increase water interaction with collagen, could be a response to the loss of water retention capacity elsewhere in the dermal extracellular matrix.
5.5 Conclusions

The aim of this chapter was to identify potential markers of ageing in human skin, this was to be performed with by assessing the existence of previously identified markers of ageing in the simplified glycated tissue models while observing for new markers presented in ex-vivo tissue samples.

The first objective was to prepare human tissue samples for characterisation using nanoscale techniques identified in chapter 2. This was achieved by optimising the cryosectioning technique and staining to show regions of interest (in this case, the reticular dermis, rich in type-I collagen). Building upon the wealth of knowledge in the field of histology, the tissue samples produced provided ample opportunity to probe morphological and nanomechanical properties.

The second objective was to apply the tools and techniques developed in chapter 4 to human skin. This included characterising the morphology of dermal collagen at the microscale and nanoscale. Disadvantages of microscale analysis with SEM were identified, as well as imaging artefacts posed by the technique. Nanoscale imaging was performed and morphological markers of ageing identified. Nanoindentation was performed on dermal collagen with results supporting the water retention hypothesis presented in the previous chapter. SMFS of dermis was not carried out due to the lack of imaging ability prior to selection of spectroscopy locations. Due to skin being a multicomponent tissue, it would have been impossible to tell what was being probed.

This study is the first to identify using AFM unique nanoscale morphological differences found in collagen fibril structure as function of ageing in skin. In addition, this study was the first to observe an age dependent decrease in the transverse stiffness of collagen fibrils. It is proposed that the properties of collagen in skin are responsive to adapt to
changes in other major components of dermal extracellular matrix to preserve its functional properties. Further studies are required to characterise water retention using other techniques as well as investigate this phenomenon in other collagenous tissues.

As discussed earlier, there are multiple processes that the various components of the dermis undergo during ageing. This study focussed specifically on changes to collagen fibril morphology and mechanical properties from accumulation of AGE products. The impact however of the other age dependent processes on the results in this study are not known.

6 Investigation of ageing of tendon

6.1 Introduction

The aim of this chapter is to further expand investigation of markers of ageing on wider native tissue samples. After identifying common markers of ageing among in-vitro glycated tissue samples and human dermis, it was decided to investigate a very different collagenous tissue. Achilles and anterior tibialis tendon were selected due to their long half-lives. Another factor distinguishing tendon from dermis is its the lack of exposure to sunlight. This removes a form of extrinsic ageing factor, reducing the complexity of changes undergone by the tissue as a function of age.

The research aim will be addressed using the following objectives;

1. Prepare Achilles and anterior tibialis tendons for characterisation using nanoscale techniques.
2. Apply the set of nanoscale characterisation tools and techniques developed in previous chapters to the Achilles and anterior tibialis tissues and introduce new techniques to support evidence of biomarkers presented thus far.

Achilles tendon and anterior tibialis tendon differ among themselves in terms of their function; Achilles tendon is a load bearing tendon, while anterior tibialis is a positional tendon. This study will examine the nanoscale effect of AGE accumulation of both types of tendon, presenting a more thorough understanding of ageing in context of the function of tissues. Presented in the following sections are some challenges faced in addressing the first objective of this chapter.

6.2 Preparing tendon for application of techniques

<table>
<thead>
<tr>
<th>Donor ID</th>
<th>Tendon</th>
<th>Gender</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Achilles</td>
<td>Male</td>
<td>14</td>
</tr>
<tr>
<td>2</td>
<td>Achilles</td>
<td>Female</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>Achilles</td>
<td>Female</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>Achilles</td>
<td>Male</td>
<td>16</td>
</tr>
<tr>
<td>5</td>
<td>Achilles</td>
<td>Female</td>
<td>19</td>
</tr>
<tr>
<td>6</td>
<td>Achilles</td>
<td>Male</td>
<td>21</td>
</tr>
<tr>
<td>7</td>
<td>Achilles</td>
<td>Male</td>
<td>65</td>
</tr>
<tr>
<td>8</td>
<td>Achilles</td>
<td>Female</td>
<td>66</td>
</tr>
<tr>
<td>9</td>
<td>Achilles</td>
<td>Female</td>
<td>68</td>
</tr>
<tr>
<td>10</td>
<td>Achilles</td>
<td>Male</td>
<td>73</td>
</tr>
<tr>
<td>11</td>
<td>Achilles</td>
<td>Male</td>
<td>74</td>
</tr>
<tr>
<td>12</td>
<td>Achilles</td>
<td>Female</td>
<td>81</td>
</tr>
<tr>
<td>2</td>
<td>Anterior tibialis</td>
<td>Female</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>Anterior tibialis</td>
<td>Female</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>Anterior tibialis</td>
<td>Male</td>
<td>16</td>
</tr>
<tr>
<td>5</td>
<td>Anterior tibialis</td>
<td>Female</td>
<td>19</td>
</tr>
<tr>
<td>6</td>
<td>Anterior tibialis</td>
<td>Male</td>
<td>21</td>
</tr>
</tbody>
</table>
As with dermis, samples were collected from healthy control donors, cryosectioned and stained as described in chapter 3. Although much was learnt about the preparation of ex-vivo tissue samples for nanoscale characterisation, there were still a number of challenges faced as described in the following sections. Table 1 provides basic information about the 12 healthy donors who took part in this study.

<table>
<thead>
<tr>
<th></th>
<th>Anterior tibialis</th>
<th>Gender</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td></td>
<td>Male</td>
<td>65</td>
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<tr>
<td>8</td>
<td></td>
<td>Female</td>
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<td>11</td>
<td></td>
<td>Male</td>
<td>74</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>Female</td>
<td>81</td>
</tr>
</tbody>
</table>

Table 1: Volunteer information for Achilles and anterior tibialis samples
Cryosectioning without cryo-embedding medium

In order to simplify the cryosectioning process and remove the number of chemical agents tissue is exposed to, the cryosectioning of tendon without embedding medium (Cryo-m-bed) was trialled. Histology sections, stained with Haemotoxin and Eosin were imaged and AFM imaging performed to observe the effectiveness of the technique. As shown in figure 6.1(a), an 8 µm section of Achilles tendon from a young donor (15) without embedding was of poor quality, with very little tissue being extracted from the bulk dissected section. AFM imaging needs little tissue for imaging due to the small scan size, so AFM imaging was performed to assess whether the little tissue that was extracted was viable for further analysis. As shown in figure 6.1(b), the sectioning was ineffective in producing smooth cross-sectional samples with little damage to the sample. Instead, there was significant shredding of fibrils, leading to widespread damage of the fibrillar matrix (*). Upon comparison of both images with figures 6.1(c) and (d), one can observe the difference made by embedding the tendon section prior to cryosectioning. The histology section, stained in Pico-sirius shows the increased content of collagen sectioned, looking relatively unaffected by the cryo-sectioning process. Further analysis with AFM imaging shown in figure 6.1(d) shows clearer, more aligned fibrillar structure (**) with fewer artefacts induced in the image. Although there still existed the presence of shredded fibrils, this was minimised. Sections were embedded in Cryo-m-bed as in the previous chapter for further analysis.
Rinsing of sections

Keeping in line with the aim of reducing exposure of tissue to chemical agents, the protocol for Pico Sirius staining was adapted to rinse the samples with water rather than wash with ethanol and then xylene. The results of this modification of procedure are shown in figure 6.2. The AFM image of a section of Achilles tendon from a 65 year old, shows features on the surface of the fibrils (*). Such features are due to residues from both the Pico Sirius stain and the Cryo-m-bed medium (Pico Sirius was visible to the

Figure 6.1: H&E (a) and pico-sirius red (c) stained cryosection of Achilles tendon from 15 year old volunteer. Sectioned without cryo-embedding medium (a,b) and with cryo-embedding medium (c,d) with AFM image of fibril matrix in both cases (b,d). * marks damage to fibrillar matrix, ** shows aligned fibrillar structure

6.2.2 Rinsing of sections

Keeping in line with the aim of reducing exposure of tissue to chemical agents, the protocol for Pico Sirius staining was adapted to rinse the samples with water rather than wash with ethanol and then xylene. The results of this modification of procedure are shown in figure 6.2. The AFM image of a section of Achilles tendon from a 65 year old, shows features on the surface of the fibrils (*). Such features are due to residues from both the Pico Sirius stain and the Cryo-m-bed medium (Pico Sirius was visible to the
naked eye as excessive powder-like red coating and Cryo-m-bed residue visible as a transparent film around the edge of the tissue section mounted on the glass slide).

Due to the deterioration in sample quality, it was decided that a rinsing procedure was essential. Rather than both ethanol and xylene vigorous rinses, a simple wash with ethanol sufficed to remove most residue left by the stain and embedding medium. The results of this process are displayed in the following section.

![AFM image of 65 year old Achilles tissue section with inadequate rinsing. * marks surface features](image)

Figure 6.2: AFM image of 65 year old Achilles tissue section with inadequate rinsing. * marks surface features

### 6.3 Results

#### 6.3.1 Histology

**6.3.1.1 Components of tendon**

Beginning with histological analysis, there are two immediate features of tendon which strike out. Firstly, tendon tissue has a very low cell density, as shown by the H&E stained Achilles tendon section, from a young donor, in figure 6.3(a). The presence of cells (indicated by dark blue dots, staining nuclei) are not observed to be common features of the bulk tendon matrix. Although tenoblasts are present at fibre boundaries as shown by
the arrows in figure 6.3(a), they are typically aligned in lines and are easily avoidable by AFM probing. The Pico Sirius red stained Achilles tendon section presented in figure 6.3(b) shows the rich collagen content of the tendon, with type-I collagen the overwhelmingly major component of the tissue matrix. The second differentiating observation is the degree of alignment of higher order collagen structure, in this case fibres and bundles of fibres (fascicles). The presence of highly ordered collagen fibres at this scale is a marker of healthy, functioning tendon.

6.3.1.2 Histology of age related changes in Achilles tendon

Interestingly, the observation of age related changes on the microscale was not obviously apparent in either Achilles or anterior tibialis tendon. Figure 6.4(a) and (b) display Achilles tendon sections from young donors and (c) and (d) from old donors. Both sets of samples show similar features; the collagen matrix is dense, with Pico Sirius red staining showing rich collagen content. The fibres and fascicles are also highly aligned with little signs of porosity in the older donor samples. One feature that was observed to change
with age was the “crimp” pattern of collagen fibres. Crimp patterns are well known periodic patterns forming a wave-like morphology along collagen fibres (Legerlotz, Dorn et al. 2014) in fibrous tissue, such as tendon (Gathercole and Keller 1991, Franchi, Ottani et al. 2010). Observing higher magnification light microscopy images of 14 year old (e) and (f) 65 year old Achilles tendon exhibits this pattern. The pattern is more pronounced in (e) than (f), and this is representative of the wider sample range. Crimping was found to be more pronounced in younger tissue, a feature that has been reported in literature (Legerlotz, Dorn et al. 2014).

Looking at anterior tibialis samples, similar features are observed for both young and old donor samples displayed in figures 6.5(a)(b) and (c)(d), respectively. There is little visible difference between older and younger tissue samples in terms of matrix density and porosity. There is however, a difference in the crimp structure as shown by (e) and
(f), again with the younger tissue (*), aged 16, exhibiting a more pronounced crimp pattern than the older tissue (**), aged 65.

![Figure 6.5: Pico Sirius red stained sections of anterior tibialis tendon from 16 (a), 19 (b), 68 (c) and 74 (d) year old volunteers. Light microscopy of anterior tibialis tendon from 16 (e) and 65 (f) year old volunteers. * marks crimp structure for younger tissue, ** marks crimp structure in older tissue.]

### 6.3.2 Nanoscale morphology

#### 6.3.2.1 AFM imaging

Surprisingly, AFM imaging of both Achilles (figure 6.6) and anterior tibialis (figure 6.7) tendon revealed little in the form of age related changes. The overall fibrillar structure can be described as fully formed, mature, fibrils exhibiting D-band periodicity, in strong alignment with neighbouring fibrils (*). The collagen matrix is dense, with no porosity and consists of large bundles of fibrils, such as those in figures 6.6(b) and 6.7(a), overlapping each other. The imaging technique is highly sensitive to debris on the sample surface, with shredding of collagen fibrils also being a common observation. Such shredding is random and not overarching in any particular cohort (**). The shredding is
an unfortunate, unavoidable artefact caused by the cryosectioning technique, though much improved compared to figure 6.1.

Figure 6.6: AFM images of Achilles tendon from 15 (a), 16 (b), 66 (c) and 74 (d) year old volunteers. * marks strong alignment, ** marks random fibril shredding.
Electron microscopy was shown in chapter 5 to be an excellent tool for gauging microscale morphology of dermal tissue. It did however introduce highly destructive desiccation cracks into the fibrillar matrix, adding to the porosity already observed in the tissue. Furthermore, SEM did not provide much more insight into nanoscale collagen structure than AFM. One interesting observation brought about by AFM imaging of...
dermis was the existence of fibril swelling in older tissue, shown by topographical line profiles across the width of the fibril. The pitfalls of this technique were many, and so TEM was introduced into this study to measure fibril diameter, d.

Presented in figures 6.8(a) and (b) are representative images of the cross-section along fibrils from a 19 (a) and 66 (b) year old Achilles tendon tissue. The cross-sections of the fibrils are circular in shape, with a large variance in their diameter. Assessment of the distribution of diameters shown in figures 6.8(c) and (d) of all old (N=1475) and young (N=1089) fibril diameters measured shows an interesting difference. The fibril diameter for old tissue, d_{old}, was found to be on average larger than the fibril diameter for young tissue, d_{young}. With d_{young}= 71.9±1.0 nm and d_{old}=85.3±1.1 nm, the difference was statistically significant using a two-way two tailed Student’s t-test (p<0.001).

![Figure 6.8: TEM images of Achilles tendon from 19 (a) and 66 (b) year old volunteers. Histograms showing distribution of collagen fibril diameters from young (c) and old (d) volunteers.](image-url)
TEM of anterior tibialis was similar to Achilles tendon in structural appearance as shown by the representative images in figures 6.9(a) and (b). The fibril cross-sections were circular and the matrix was densely packed.

For anterior tibialis tendon this difference in fibril diameter between young and old tissue was more prominent as shown in figures 6.9(c) and (d). There is a distinct tail in the distribution of $d_{\text{old}}$, with the population containing a large proportion of large diameter fibrils. This was reflected in statistical analysis (two-way Student’s t-test); with $d_{\text{young}}=72.0\pm1.7$ nm (N=600) and $d_{\text{old}}=100.1\pm1.7$ nm (N=818) with two tailed t-tests showing the two populations as significantly different ($p<0.001$). Interestingly, fibril diameter for both sets of young tissues (Achilles tendon and anterior tibialis) had similar fibril diameters, with no statistically significant difference between the two populations ($p=0.93$). Fibril diameters for both sets of old tissues (Achilles tendon, $d_{\text{old,AT}}=85.3\pm1.1$ nm and anterior tibialis, $d_{\text{old,ATT}}=100.1\pm1.7$ nm) were found to be statistically significant ($p<0.001$).
Nanoindentation

The distributions of elastic modulus, $E$, measured for all Achilles tendon tissue samples are displayed by the stacked histogram in figure 6.10(a). The distributions vary greatly, within the young and old cohorts. Upon viewing the boxplot in figure 6.10(b), it is not immediately clear if there is an age related trend in $E$, with neither cohort displaying distinct features in elastic modulus. Once all data from old (c) and young (d) tissue are pooled, the overall distributions of both cohorts are very similar. The mean elastic modulus for young tissue was found to be, $E_{\text{young}}=6.58\pm0.17$ GPa while for old tissue, $E_{\text{old}}=7.41\pm0.18$ GPa ($p=0.0009$). While the difference is statistically significant (two way

Figure 6.9: TEM images of anterior tibialis tendon from 19 (a) and 74 (b) year old volunteers. Histograms showing distribution of collagen fibril diameters from young (c) and old (d) volunteers.
Student’s t-test, it is marginal and not strong. However, we can reject the null hypothesis and conclude that elastic modulus of fibrils from Achilles tendon increased with age.

Considering the elastic modulus, of anterior tibialis tendon tissue samples, there is a greater difference between young and old tissue samples as shown in figure 6.11. The distributions of E as a function of age present no obvious trend as shown in histograms from figure 6.11(a). Distributions are non-parametric and do not show a clear difference between the two cohorts. Boxplots shown in figure 6.11(b) begin to show a reduction in the variance and means of E from older tissue. Pooling data for all old and all young as shown in figures 6.11(c) and (d) respectively, a clear difference between the two groups – with young tissue exhibiting a larger proportion of fibrils with a high elastic modulus. The mean elastic modulus for young tissue was $E_{\text{young}} = 11.67 \pm 0.29 \text{ GPa}$ (N=729) while for old tissue, $E_{\text{old}} = 8.44 \pm 0.30 \text{ GPa}$ (N=828, p<0.001). The strong statistical significance (two-way Student's t-test) helps to inform the relationship between age and elastic modulus of fibrils from anterior tibialis tendon. The elastic modulus of anterior tibialis tendon fibrils decreased with age.
Figure 6.10: Histograms (a) and boxplots (b) showing distributions of $E$ of collagen fibrils from Achilles tendon from various aged volunteers. Histograms showing distribution of $E$ of collagen fibrils from old (c) and young (d) volunteers.
Figure 6.11: Histograms (a) and boxplots (b) showing distributions of $E$ of collagen fibrils from anterior tibialis tendon from various aged volunteers. Histograms showing distribution of $E$ of collagen fibrils from old (c) and young (d) volunteers.
6.3.4  Single molecule mechanics (SMFS)

Investigating the contour length of molecules from Achilles tendon showed no observable
differences in the distribution of $L_0$ as shown by histograms in figure 6.12(a) and boxplots
in figure 6.12(b). Upon pooling $L_0$ for old and young tissue, the histograms showing
distributions in figures 6.12(c) and (d) respectively, showed no obvious change with age.
This was further supported by statistical analysis (Student’s t-test) which found that the
mean $L_0$young$=78.32\pm1.78$ nm (N=1102) and $L_0$old$=73.07\pm2.74$ nm (N=553) and p=0.1,
showing no statistically significant difference. It can therefore be concluded that the
contour length of collagen molecules from young Achilles tendon tissue and from old
Achilles tendon issue do not vary.

In contrary to this, the contour length of molecules from anterior tibialis tendon showed
a slight decrease in the distributions of $L_0$ as shown by histograms in figure 6.13(a) and
boxplots in figure 6.13(b). Pooling $L_0$ for old and young tissue showed a clear difference
in their distributions as shown by histograms in figure 6.13(c) and (d). Statistical analysis
showed $L_0$young$=97.80\pm1.78$ nm (N=839) and $L_0$old$=83.30\pm4.03$ nm (N=384) with
p<0.001, showing strong statistical significance. It can therefore be concluded that the
contour length of collagen molecules from old anterior tibialis tissue was smaller than
the contour length of collagen molecules from young anterior tibialis tissue.

There were a number of tissue samples irrecoverably lost during this experiment for both
tendon types. This was due to the rinsing procedure weakening the adsorption of tissue
to the glass slide substrate. The result was an increased likelihood of sample
delamination, which occurred for a number of samples once submerged for SMFS.
Figure 6.12: Histograms (a) and boxplots (b) showing distributions of $L_0$ of collagen fibrils from Achilles tendon from various aged volunteers. Histograms showing distribution of $L_0$ of collagen fibrils from old (c) and young (d) volunteers.
Figure 6.13: Histograms (a) and boxplots (b) showing distributions of $L_0$ of collagen fibrils from anterior tibialis tendon from various aged volunteers. Histograms showing distribution of $L_0$ of collagen fibrils from old (c) and young (d) volunteers.
6.4 Key findings

6.4.1 Morphological markers

6.4.1.1 Microscale markers

A feature of high order fibrillar collagen structure was observed in tendon, unlike in other tissue or in-vitro models from previous chapters. The formation of wave-like patterns, known as crimping of collagen fibres was observed with light microscopy. The crimping pattern was found to be more prevalent in younger tissue, providing a marker of ageing at the macroscale. Unlike in dermis, there was no observable loss of collagen matrix density or increased porosity within tendon tissue due to ageing. The crimping pattern has been reported in literature (Gathercole and Keller 1991, Legerlotz, Dorn et al. 2014), thought to be apparent in the natural state of the tendon, and in this study was found for both Achilles tendon and anterior tibialis tendon. It is unclear however how this is related to the accumulation of AGEs and so crimping can be considered an independent ageing process to the focus of this study.

6.4.1.2 Nanoscale markers

Unlike in previous chapters, AFM imaging did not show any differences between young and old tissue, for either Achilles tendon or anterior tibialis tendon. Amid the high alignment and packing density of collagen fibrils in tendon, accumulation of AGE cross-links did not seem to impact on these characteristics of tendon.

In this chapter, TEM was introduced to measure fibril diameters in order to determine whether fibrils did indeed swell with the age of the tissue. A key finding was the increase in fibril diameter with age for both Achilles tendon and anterior tibialis tendon, with anterior tibialis tendon making a more significant increase with age. This shows the density of collagen fibrils decreases with age, in line with the fibril hydration hypothesis.
proposed in the earlier chapters. Interestingly, no loss of D-banding was observed by AFM imaging which is usually a characteristic of fibril hydration.

This finding is of especial interest due to the nature of the tendons themselves. Achilles tendon is a load bearing tendon (Birch 2007). Cyclical loading increases collagen fibril diameter due to lateral fusion of fibrils, which leads to the expectation that fibril diameter of Achilles tendon would increase too. However, this study has found anterior tibialis, a positional tendon under far less strain (Birch 2007), was found to increase fibril diameter with age. This implies the increase is not due to loading, but due to the increased AGE concentration within the tissue, leading to increased fibril hydration. Anterior tibialis provides a more simplified case of the impact of AGEs on tendon. It may be possible that load bearing undergone by Achilles tendon disrupts the impact of AGEs on water retention within fibril and thus fibril swelling.

6.4.2 Nanomechanical markers

Nanoindentation of Achilles tendon yielded very interesting results, with fibrils showing a slight increase in the elastic modulus with age. This result however was marginally statistically significant.

Fibrils from anterior tibialis were shown to undergo a significant decrease in elastic modulus with age, leading to the conclusion that fibril density had decreased. This supports the findings of the TEM diameter measurements. As with previous chapters, this finding supports the proposed theory that increased AGE concentration with age increases the fibril’s ability to retain water, thus causing swelling and decreasing its transverse stiffness.
Considering the SMFS experiment it was of special interest to find that fibrils from young and old Achilles tendon showed no statistical difference in contour length. This implies accumulation of AGEs (including AGE cross-links) is not having an effect on the binding affinity between collagen molecules.

Fibrils from young and old anterior tibialis tendon however, showed significant statistical difference, with a decrease in contour length occurring for older tissue. This increased binding affinity between collagen molecules is due to the accumulation of AGE cross-links with age.

6.4.3 Is adaptive ageing present in anterior tibialis tendon?

Similar to dermal tissue, tendon is also a multi-component tissue (though not to the same extent) with various important extracellular components. Proteoglycans such as decorin, hyaluronic acid, biglycan, fibromodulin, lumican, epiphycan and keratocan are present in tendon (Iozzo and Murdoch 1996, Derwin, Soslowsky et al. 2001), attaching across collagen fibrils (Scott, Orford et al. 1981). It is known however that they decrease in concentration with tissue age (Scott, Orford et al. 1981). Proteoglycans in tendon provide the ability to resist force and strain due to compression and tensile stretching. With a loss of such proteoglycans corresponding with an increase in the concentration of AGEs in tissue, the question is raise about what role AGEs may have if any, on counteracting the loss of proteoglycans from the tendon extracellular matrix.

6.5 Conclusions

This chapter aimed to identify potential nanoscale markers of ageing of human tendon. The objectives set to achieve this involved preparing ex-vivo Achilles tendon and anterior tibialis samples for characterisation using the methodology adapted from previous
chapters. Although this met several challenges, preparation of cryosections from conventional histology techniques were optimised specifically for the two types of tendon. Tendon samples were able to be investigated with all methodologies developed. The second objective was to identify potential markers of ageing using the techniques developed. Both types of tissue presented interesting results with anterior tibialis tendon displaying similar markers of ageing as dermis and the in-vitro model. This included a decrease in the elastic modulus of collagen fibrils as a function of age and an increase in the fibril diameter (swelling). The implication of such findings support the AGE induced fibril hydration mechanism proposed in previous chapters. Such findings also corresponded with a decrease in contour length, and so an increase in the binding affinity between collagen molecules, a direct result of the accumulation of AGE cross-linking. The study found that Achilles tendon however did not undergo similar changes, possibly due to its role as a load bearing tendon, however, ageing of Achilles tendon in this context requires further exploration.

This is the first study to take a comprehensive look at localised nanomechanical properties of collagen in tendon as a function of ageing. This differs greatly from previous studies which focussed solely on bulk mechanical properties, investigating tensile strength of fibres rather than looking deeper at the more fundamental level of collagen fibrils and indeed molecules. It is also the first to propose that Achilles tendon undergoes different impact of ageing compared to anterior tibialis tendon, which is more similar to dermis and the in-vitro glycated model.
7 Synopsis

7.1 Why ageing?

Ageing is a topic that everyone has a stake in. As described in chapter 1, there is no single process of ageing, instead a culmination of a number of deleterious processes affecting the functioning of eventually, the entire body. This study has focussed on connective tissue in particular as it is present almost everywhere in the body, providing structural integrity. Collagen in particular is the major structural protein and has unique properties making it the building block of a variety of tissues.

Most work to date investigating the impact of ageing on connective tissue has been performed on bulk tissue, assessing it as a system rather than examining individual components such as fibrillar collagen. So in this thesis, I set out to investigate specifically what happens to collagen as we age. By doing so at the nanoscale I was able to isolate from the complex system of tissue, the impact of one particular ageing process on the properties of fibrillar collagen.

7.2 Addressing of the research question

In my original research question, I set the aim to investigate the impact of AGEs on the mechanobiology of collagen, the major structural protein in connective tissue extracellular matrix. The objectives set for this were to firstly identify suitable non-bulk methodologies to investigate impact of AGE accumulation at the nanoscale. Secondly to develop an in-vitro model of ageing to assess impact of AGEs in a simplified manner. Thirdly to apply the methodologies, tools and techniques developed for the in-vitro model to wider ex-vivo tissue samples to assess physiological relevance and identify biomarkers of ageing.
In chapter 2, I identified nanoscale techniques through a comprehensive literature review of various candidates for consideration. In chapter 4a I developed successfully an in-vitro glycated tissue model which could be used to isolate the impact of AGE accumulation from other natural processes within collagenous tissue. There was a lack of connective tissue models to mimic the ageing process effectively and so this was a crucial stage in my study. In chapter 4b I applied tools and techniques identified in chapter 2 to find successfully nanomorphological and nanomechanical markers of ageing in fibrillar collagen. In chapter 5 I expanded the study to ex-vivo tissue samples. I prepared dermis for application of the methodology and identified similar nanomorphological and nanomechanical markers of ageing in dermal collagen. In chapter 6 I expanded the ex-vivo study further, preparing Achilles and anterior tibialis tendon for the application of my methodology. I identified similar nanomechanical markers of ageing in anterior tibialis tendon, however was unable to do so for Achilles tendon. I explored reasons why Achilles tendon did not display similar features with ageing, however it is still inconclusive and warrants further investigation.

7.3 My novel contribution

My novel contribution is the hypothesis that accumulation of AGEs is part of an adaptive ageing process within tissue, to counter balance some deleterious processes within the system of the tissue (such as the loss of hydrophilic components), in order to retain its functional properties. This hypothesis suggests that AGEs introduce a new water interaction mechanism of collagen, leading to the ability of collagenous tissues to retain hydration with the accumulation of AGEs. My hypothesis has led to wider computational modelling studies which have shown the water interaction mechanism to be energetically favourable to glucosepane (Ahmed, Nash et al. 2017).
7.4 Limitations of the study

There were various limitations identified of this study, which were not addressable within the scope of this work;

The effect of full sample hydration on collagen mechanical properties was not investigated for the in-vitro or tendon samples as it was for dermis. One reason why is the difficulty to image and nanoindent in-vitro samples, as a hydrogel it would be highly absorbent, turning into a porous network of fibrils. It is also unknown what the exact physiological level of hydration (relative humidity) is for various tissues. It is unlikely to be 100% as was the case for the hydrated dermis samples and so this wider investigations into this was not in the scope of this study.

Fibril diameter measurements using TEM were not carried out on all samples and so fibril swelling could not be quantified in dermis and in-vitro model. TEM could not be used on in-vitro model or skin due to the random orientation of collagen fibrils and sheets. Cross-sections across randomly oriented collagen would result in inaccurate diameter measurements. AFM line profiles are not an accurate measure of fibril width, however alluded to changes in fibril diameter in dermal tissue.

The concentration of glycating agents was not increased with time. The impact of this was alluded to in chapter 4 as perhaps explaining the change in nanomechanical properties in methylglyoxal glycated gels. The glycation media was not disturbed in an attempt not to disturb the experiment or introduce bacterial contamination, however the effect of reagent concentration is important to investigate.

7.5 Future work

It is important to develop the in-vitro model to mimic more accurately the in-vivo environment. This can be done in numerous ways, adding a wealth of different
components to the model (such as cells, elastin, proteoglycans, etc), however the most important of which is to develop a sealed glycation bioreactor to keep glycating agent constant or increasing.

In terms of augmenting the ex-vivo tissue studies, increasing the number of tissue samples is of importance. This was difficult in the scope of my project as samples were donated on a voluntary basis (with pain and scarring being limiting factors of volunteer recruitment). Expanding the ex-vivo dermis study can be carried out by examining different sections of the dermis, incorporating the papillary dermis and the dermal-epidermal junction. A larger scope of ex-vivo study including further collagen rich tissues (e.g. cartilage, lens, vascular tissue, bone) will also refine a better understanding of the role of collagen and AGEs.

It is important to improve characterisation of AGEs, identification and quantification, using techniques such as mass spectroscopy and high performance liquid chromatography. This was beyond the scope of this project, however a potential collaborative project. A simple direct measurement of the presence of AGEs can be performed using fluorescence microscopy. As described in chapter 1, AGEs such as pentosidine autofluoresce. Although glucosepane, the AGE of most interest doesn’t, presence of fluorescence in in-vitro glycated models is an indication of successful AGE formation. A more direct measure and quantification of increased hydration within fibril is also an important aspect to explore. DSC was trialled, however it is very difficult to control the interstitial water content within in-vitro tissue models, furthermore as described previously, the level of hydration in native tissue is not known.
There are many wider societal impacts of this research, due to the overarching relevance of ageing on all. This study shows ageing is not restricted to impacting just a cellular process, or causing a deterioration in genetic coding. It is also a biochemical process, intrinsic, and endogenous. While it may seem unstoppable, there are perhaps ways to counter the effects with many studies having investigated potential AGE cross-link breakers and inhibitors (Bakris, Bank et al. 2004)-(Asif, Egan et al. 2000). The promotion of hydration in tissues such as skin with cosmetic products to counteract the loss of proteoglycans and aid AGEs in increasing extracellular matrix hydration is a topic of interest, with hyaluronic acid being a candidate component with hydrophilic properties. Furthermore research into advanced glycation end products is not only relevant in the context of ageing, but also highly relevant in diabetes, in which hyperglycaemic conditions greatly accelerate accumulation of AGEs. We have only scratched the surface of understanding advanced glycation end products and their impact on tissue. Continued research into this field may lead us to one day be armed with a host of products blocking the effect of glycation or even perhaps reversing it.
8 References


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203


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