Design and Formulation of an \textit{In-situ} Gelling Collagen for Tissue Specific Modified Drug Release and Cell Therapy

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I, Saja Muwaffak 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.
Abstract

Much research is dedicated to developing biomedical materials that can be utilised by minimally invasive techniques. Collagen is a good candidate for further development. It was hypothesised that an injectable collagen hydrogel could be developed for use in drug delivery and pharmaceutical research.

Initial experiments examined different collagen and hyaluronic acid (HA) hydrogels. Collagen type I and tyramine substituted HA hydrogels were prepared and characterised mechanically and by in-vitro 3D cell cultures. The possibility of formulating an injectable artificial vitreous substitute was studied by photochemical crosslinking of HA and collagen. A potential vitreous substitute was prepared using riboflavin 5'-phosphate and blue light at 4 mW/cm². The in-situ preparation of the vitreous substitute showed a significant reduction in cell viability in ARPE-19 cells due the generation of reactive oxygen species (ROS). However, there may be potential to use the crosslinked material as a simulated vitreous fluid for pharmaceutical research.

A lyophilisable in-situ polymerisable collagen (IPC) designed for injectability with tuneable mechanical properties (500 - 2500 Pa) was then prepared. The IPC was prepared by dialysis of collagen type I against EDTA following an optimised process. Solid drug powder IPC gel formulations of the anti-scarring agents: ilomastat and AZ 8955 were investigated to mediate healing after eyelid surgery for trichiasis. A 100 µL depot which could potentially be injected into the subconjunctival space at the surgical site in the eyelid achieved a 2-week in vitro release profile at therapeutic concentrations. Drug release profiles were dependent by depot volume or the drug loading. Drug release profiles were primarily dependent on drug solubility. Poorly soluble drugs were mixed with the IPC as powder suspensions for the purpose of localising drug in the tissue and to minimise the risk of localised foreign body reactions. A moderately soluble anti-scarring drug, pirfenidone was mixed into the IPC as a solution but release was too rapid, so pirfenidone was pre-encapsulated into PLGA particles by an electrospray process and then incorporated into the IPC.

The antibody, bevacizumab which is widely used to treat ocular angiogenesis and can mediate post-surgical healing was also formulated with
the IPC and displayed a release profile of 30 days in the presence of high molecular weight HA (2 mg/mL).

The IPC was also utilised as a vehicle to establish a method for preparing an injectable cellular delivery formulation using human tenon fibroblasts (HTF). IPC-based 3D cultures of HTF cells showed excellent cell viability (>90%) and attachment after 7 days.
Impact Statement

The focus of this PhD project was to study the therapeutic applications of collagen-based biomaterials for several biomedical applications. Notably, this work presents the advantageous properties of these advanced collagen biomaterials within the context of the realm of ophthalmology. However, there are also clear clinical applications for the formulations elsewhere, including other medical fields (e.g. cancer, lung pathologies). The injectable in-situ polymerisable collagen and other collagen-based hydrogels were conceived and designed a drug delivery depot for intraocular administration, but also may have potential for other routes of administration such as subcutaneous injection.

The preparation of an artificial vitreous substitute could provide a platform for pharmaceutical and biomaterial scientists to screen and evaluate various drug candidates and formulations for biomedical applications. Secondly, the IPC hydrogels that were optimised and developed in this work could appeal to scientists in the pharmaceutical and regenerative fields where prolonged and localised drug release is desirable for improved therapeutic outcomes. More specifically, there is a real clinical need for this type of formulation for the treatment of trichiasis of the eye where clinical options are very limited.

Importantly, there are several and quite interesting findings described in the thesis. For instance, the IPC was successfully formulated with potent anti-scarring agents and showed a clinically favourable drug release profile. Moreover, the formulation was found to not affect the cell viability in human eye cells yet remained bioactive after 7 days of release by resulting in a similar level of inhibition of HTF embedded collagen contraction to the control. In addition, in combination with hyaluronic acid, the IPC formulation also achieved a 30-day release profile of a monoclonal antibody which is an entirely different therapeutic drug class. Finally, a cell-IPC formulation method gave encouraging results for cell viability and which could be taken forward for additional development for cell therapeutic applications.

It is expected that the work described in this Thesis could potentially direct advances in collagen gel-based therapies for localised delivery of
therapeutics. The key results in this thesis have also been communicated in national and international conferences and will be published in appropriate peer review journals.
Acknowledgment

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<td>AMD</td>
<td>Age-related macular degeneration</td>
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<tr>
<td>AS</td>
<td>Acid Soluble Collagen</td>
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<tr>
<td>AIPc</td>
<td>Al(III) Phthalocyanine chloride disulfonic acid (adjacent isomer)</td>
</tr>
<tr>
<td>ARPE-19</td>
<td>Human retinal pigment epithelium cell line</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<td>Basic fibroblast growth factor</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential Scanning Calorimetry</td>
</tr>
<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>F-ANOVA</td>
<td>Factorial ANOVA</td>
</tr>
<tr>
<td>F7</td>
<td>Rat schwannoma cells</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FT-IR</td>
<td>Fourier-transform infrared spectroscopy</td>
</tr>
<tr>
<td>GAGs</td>
<td>Glycosaminoglycans</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HA-T</td>
<td>Hyaluronic acid tyramine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>HDFa</td>
<td>Human dermal fibroblast cells</td>
</tr>
<tr>
<td>HTFa</td>
<td>Human Tenon Fibroblasts adult</td>
</tr>
<tr>
<td>IPC</td>
<td>In-Situ Polymerisable Collagen</td>
</tr>
<tr>
<td>IPF</td>
<td>Idiopathic Pulmonary Fibrosis</td>
</tr>
<tr>
<td>IVT</td>
<td>Intravitreal</td>
</tr>
<tr>
<td>GAGs</td>
<td>Glycosaminoglycans</td>
</tr>
<tr>
<td>LCST</td>
<td>Lower critical solution temperature</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum Essential Medium</td>
</tr>
<tr>
<td>MES</td>
<td>4-Morpholineethanesulfonic acid</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloprotease</td>
</tr>
<tr>
<td>MMPi</td>
<td>Matrix Metalloprotease inhibitor</td>
</tr>
<tr>
<td>Mw</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NHS</td>
<td>N-Hydroxysulfosuccinimide sodium salt</td>
</tr>
<tr>
<td>ORM-ANOVA</td>
<td>One Way Repeated Measures ANOVA</td>
</tr>
<tr>
<td>PAAc</td>
<td>Poly(acrylic) acid</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PC</td>
<td>Pepsin soluble collagen</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>PEO</td>
<td>Poly(ethylene) oxide</td>
</tr>
<tr>
<td>PFA</td>
<td>4 % paraformaldehyde</td>
</tr>
<tr>
<td>PLA</td>
<td>Poly(lactic) acid</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(lactic-co-glycolic) acid</td>
</tr>
<tr>
<td>PNIPAAM</td>
<td>Poly(N-isopropylacrylamide)</td>
</tr>
<tr>
<td>PVA</td>
<td>Poly(vinyl) alcohol</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RTC</td>
<td>Rat-tail Collagen</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM*</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-buffered saline in Triton X</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>-------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>$T_d$</td>
<td>Thermal denaturation temperature</td>
</tr>
<tr>
<td>$T_g$</td>
<td>Glass transition temperature</td>
</tr>
<tr>
<td>$T_m$</td>
<td>Melting point</td>
</tr>
<tr>
<td>$T_{td}$</td>
<td>Thermal degradation temperature</td>
</tr>
<tr>
<td>TGF$\beta$</td>
<td>Transforming growth factor $\beta$</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>Tissue inhibitor of MPP 1</td>
</tr>
<tr>
<td>TNF$\alpha$</td>
<td>Tumour necrosis factor $\alpha$</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VH</td>
<td>Vitreous humour</td>
</tr>
<tr>
<td>WB</td>
<td>Washing buffer</td>
</tr>
</tbody>
</table>
Chapter 1: General Introduction
1. General Introduction

1.2 Collagen

Collagen is a protein that forms elongated fibrils that provide mechanical stability to animals. Collagen is the most abundant protein in mammals where at least 45 distinct genes are responsible for a known family of 29 different collagen proteins. Collagen is found in even the most primitive animals, including sponges, and is considered intrinsic to the evolution of metazoans \(^1\). \(^2\). In humans collagen comprises one third of the total protein, accounts for three-quarters of the dry weight of skin and is the most prevalent component of the extracellular matrix (ECM) \(^3\). Human collagens are composed of ~25 different polypeptide chains assembled in different combinations.

Collagens are trimeric proteins formed by association of three polypeptide chains. These can be identical homotrimer collagens (identical) or heterotrimer collagens (different). Different genes code for distinct collagen chains, and only chains from the same collagen type associate with each other \(^4\), \(^5\). Vertebrate collagens are designated with roman numerals (I–XXVIII) in the chronological order of their discovery. The individual polypeptide chains are named with the letter \(\alpha\) and an Arabic numeral. For instance, the cartilage-predominant type II collagen is a homotrimer of three \(\alpha1(II)\) chains, whereas the bone predominant type I collagen (Figure 1) is a heterotrimer made of two \(\alpha1(I)\) chains and one \(\alpha2(I)\) chain. Each of the different \(\alpha\) chains have different amino acid content and sequence as well as differing levels of posttranslational protein modification \(^4\), \(^6\).

Collagen can either be fibrillar or non-fibrillar and different types of collagen are classified by their function, domain architecture and supramolecular organization. The main fibril forming collagens (I, II, III, V and XI) represent 80–90% of the human collagens and are the principal source of tensile strength in animal skin, bones, cartilage, blood vessels, etc. Certain non-fibrillar collagen types form hexagonal lattices (VIII and X), beaded filaments (VI), anchoring fibrils (VII) or have transmembrane domains \(^1\), \(^7\).
1.2.1 Hierarchal Structure of Collagen

Collagens share the same basic, triple-helical structure as a defining feature. The motif portrays three parallel polypeptide strands (α chains) in a left-handed, polyproline II-type (PPII) helical conformation. The triple helix structure is held together by interstrand hydrogen bonding (Figure 1) between the amino acids in the helix. The tight packing of PPII helices within the triple helix mandates that every third residue be glycine (Gly), resulting in a repeating

Figure 1. Overview of the collagen type I triple helix structure. (A) Crystal structure of the triple helix, (B) triple helix with the three strands depicted in space-filling, ball-and-stick, and ribbon representation, (C) ball-and-stick image of a segment of the triple helix, with the ladder of interstrand hydrogen bonds and (D) hydrogen bonds, blue dashed lines. Reproduced from Shoulders and Raines 2010 [3] and Chattopadhyay and Raines [5].
Xaa-Yaa-Gly sequence, where Xaa and Yaa can be any amino acid. Glycine is essential for the formation of the collagen triple helix since it has no residue substituents so it can fit into the central core of the helix.\(^3\),\(^8\).

Hydroxyproline and proline are also essential for collagen stability as they allow for the sharp twisting of the collagen triple helix (tropocollagen). The amino acid repeat occurs in all types of collagen, although, it is disrupted at certain locations within the triple-helical domain of non-fibrillar collagens. The amino acids in the Xaa and Yaa positions of collagen are often post-translationally hydroxylated proline residues, i.e. \((2S)\)-proline(Pro) and \((2S,4R)\)-4-hydroxyproline (Hyp), respectively. Pro-Hyp-Gly is the most common triplet in collagen.\(^3\),\(^9\). Hydroxylsine is found in collagen and represents up to 2% of the amino acid sequence in collagens. Hydroxyproline and hydroxylsine appear to be found almost uniquely in collagen.\(^8\).

Fibrillar collagens assemble (Figure 2) via the lateral association of tropocollagen triple helices first into fibrils (~10–300 nm diameter) and then fibres (~1–20 μm).\(^{10}\) Fibres may further associate into large bundles, such as in tendons, with diameters of up to ~500 μm.\(^2\),\(^{11}\). The formation of fibrillar collagen follows several intricate processes in which tropocollagen first forms. The three left-handed helices are twisted together into a right-handed triple helix, a cooperative quaternary structure stabilised by non-covalent bonds. Tropcollagens associate with each other side to side to form the collagen microfibril. The side to side association forms a regular banding pattern known as D-periodicity, seen in electron microscopy. Within, the fibrils, collagen molecules of length 300 nm and width 1.5 nm are staggered with respect to their neighbours by multiples of D which can range from 64-67 nm.\(^1\),\(^3\).
1.2.2 Assembly of Fibrillar Collagens

When collagens assemble into fibrils during fibrillogenesis, the fibrils are subsequently stabilised by enzymatic and non-enzymatic crosslinks (Figure 3). Two short segments called telopeptides flank the major triple helix (at the C- and N-termini) in mature fibrillar collagen molecules which enable collagen to assemble into fibrils. Telopeptides are about 20 residues in length, at both N- and C-termini of the polypeptide chains. Telopeptides do not possess the repeating Gly-X-Y motif and are therefore non-helical, i.e. non-collagenous[1],[2].

Enzymatic crosslinking involves the action of lysyl oxidase which crosslinks the telopeptides in the triple helices. The enzyme catalyses the covalent aldol reaction between the lysine or hydroxylysine residues in the N- and C-telopeptides of adjacent molecules, thus bonding two molecules head-to-tail along the fibril[12]. The crosslinking forms hydroxylysyl pyridinoline and lysyl pyridinoline cross-links between lysine and hydroxylysine residues. The cross-linking process endows mature collagen fibrils with strength and stability. Although collagen telopeptides might not be essential for nucleating collagen fibrillation.

Figure 2. Fibrillar collagen type I assembly and organisation. Tropocollagens self-assemble in a quarter-staggered array into microfibrils to form collagen fibrils with characteristic periodic D-spacing, reproduced from Calenon and Wallace. 2016[10].
fibrillogenesis, their absence greatly weakens the mature fibrils [3]. Non-enzymatic crosslinks occur within the triple helical structure. The crosslinking process involves hydroxylation and hydroxylsine glycosylation which react with amino acids such as lysine and arginine to form glycation end products crosslinks like pentosidine [13]. Both nonenzymatic and enzymatic derived intermolecular cross-links represent the monomeric collagen content found in tissues. With age, the natural cross-links can chemically rearrange to form acid-stable aldminine cross-links to form polymeric collagen in tissues. This in turn results in an increased mechanical strength of mature tissue [14].

![Diagram](image3.png)

**Figure 3.** The pathways of enzymatic and non-enzymatic crosslink formation in fibrillar collagen. Adapted from Garnero 2012 [14].

The level and type of post translational modification vary in even the same type of collagen resulting in a varying number of collagen molecules in each fibril. This causes the fibril diameter and fibre arrangement to vary. Hence, the same collagen type can have different functions depending on the biological requirements. The interplay between different collagen types which often exist together provides the ability of further adaptation of collagen to have different biological and mechanical properties [15].

### 1.2.3 Different Collagen Types

Type I collagen is a heterotrimer consisting of two α1 (I) chains and one α2 (I) chain which form the three polypeptide chains in the triple helix (Figure 4). The amino acid content of α1(I) and α2(I) polypeptide chains that form type I
collagen (Table 1) indicate that proline-hydroxyproline-proline are by far the most prominent amino acid triplets in each chain \[^5\].

**Table 1.** The percentage of individual amino acids in each polypeptide chain in collagen type I. Adapted from Chattopadhyay and Raines 2014 \[^5\].

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Proportion in α1(I) chain (%)</th>
<th>Proportion in α2(I) chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>11.74</td>
<td>10.51</td>
</tr>
<tr>
<td>Arginine</td>
<td>5.02</td>
<td>5.30</td>
</tr>
<tr>
<td>Asparagine</td>
<td>1.23</td>
<td>2.18</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>3.13</td>
<td>2.27</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>4.92</td>
<td>4.36</td>
</tr>
<tr>
<td>Glutamine</td>
<td>2.56</td>
<td>2.27</td>
</tr>
<tr>
<td><strong>Glycine</strong></td>
<td><strong>32.67</strong></td>
<td><strong>32.77</strong></td>
</tr>
<tr>
<td>Histidine</td>
<td>0.28</td>
<td>0.76</td>
</tr>
<tr>
<td>Hydroxylysine</td>
<td>0.38</td>
<td>0.85</td>
</tr>
<tr>
<td><strong>Hydroxyproline</strong></td>
<td><strong>10.80</strong></td>
<td><strong>9.38</strong></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.85</td>
<td>1.70</td>
</tr>
<tr>
<td>Leucine</td>
<td>2.08</td>
<td>3.13</td>
</tr>
<tr>
<td>Lysine</td>
<td>3.22</td>
<td>1.99</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.66</td>
<td>0.38</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1.23</td>
<td>1.42</td>
</tr>
<tr>
<td><strong>Proline</strong></td>
<td><strong>12.03</strong></td>
<td><strong>10.23</strong></td>
</tr>
<tr>
<td>Serine</td>
<td>3.50</td>
<td>3.31</td>
</tr>
<tr>
<td>Threonine</td>
<td>1.61</td>
<td>1.89</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.47</td>
<td>0.38</td>
</tr>
<tr>
<td>Valine</td>
<td>1.61</td>
<td>3.22</td>
</tr>
</tbody>
</table>

Collagen type II triple helix (Figure 4) consists of three α1 (II) chains and forms fibrils similar in structure to type I, with a similar 67-nm periodicity. In collagen type I and II there is a difference in distribution of the triple helix-
stabilising hydroxyprolines and electrostatic and hydrophobic properties result in the differences between the collagens. [3], [16].

**Figure 4.** The structural difference in the alpha chains of type I and type II collagen. Collagen type I fibrils contain 2α1 (I) and 1α2 (I) chains. Collagen type II contains fibrils contain 3α1 (II) chains. Reproduced from Gao et al. 2017 [16].

Collagen type IV (non-fibrillar collagen) is an important collagen and has a different quaternary structure to fibrillar collagens. The domain at the protein C-terminus is not removed in post-translational processing, and the fibres link head-to-head, rather than in parallel (as in seen collagen type I). Collagen IV lacks the regular glycine in every third residue which is necessary for the tight collagen helix. This makes the overall arrangement looser with kinks allowing collagen IV to form basement membranes (Figure 5). These features allow the collagen to form in a sheet, forming the basal lamina in basement membranes [17], [18].

**Figure 5.** Collagen IV structure and alignment in basement membranes. The NC1 and S7 domains allow for the assembly of collagen into basement membranes. Reproduced from Cosgrove and Liu.2017 [18].
1.2.4 Biological Function of Collagen

Collagen has adaptable mechanical properties and is present in virtually every extracellular tissue with mechanical function. Collagen confers mechanical stability, strength, and toughness to a range of tissues from tendons and ligaments, to skin, cornea, bone, and dentin. These tissues have very different mechanical requirements, some need to be elastic while others need to primarily store kinetic energy or provide stiffness and toughness. In tendons and ligaments, collagen type I transmits force to muscles and bones resulting in the possibility of smooth walking. Collagen type I confers fracture resistance to bones and tooth dentin as it comprises most of the organic matrix of these biological structures. Muscles can also not function without the presence of the collagen-rich matrix that is present around the contractile cells [3], [5], [8].

The complex hierarchal structure of collagen leads to a great variety of properties that serve a given function. Taking the example of bones, a remarkable hierarchical biomaterial that has two major constituents, soft collagen protein and much stiffer apatite mineral (Figure 6). During the formation of bone, collagen molecules assemble into fibrils, which are mineralised via the formation of apatite crystals. The amount of apatite varies depending on the bone in different sites of the human body. Every bone in the human body will therefore have a slightly different arrangement of the mineralised collagen fibrils and different local architecture. This results in a mechanical stiffness, different bending stiffness and fracture resistance which varies depending on the body site [19]–[21].

**Figure 6.** Hierarchical structure of bone ranging from the macroscale skeleton to nanoscale collagen (green) and hydroxyapatite (red). Reproduced from Orgel et al.2006. [21]
Type-I collagen fibrils are anisotropically oriented in multiple tissues, including tendons, ligaments and the skin. The domains capable of binding to cells or special ligands in collagen are arranged in a specific manner on the collagen fibrils. \textit{In-vivo}, cells and an intricate biomolecular signalling network regulate these self-organised structures. \textit{In-vitro} approaches to create orderly aligned collagen-based biomimetic materials for tissue engineering are actively being developed \cite{22}.

Collagen type II is also a fibrillar collagen and represents a critical component of softer tissues such as cartilage. Collagen type II also constitutes the main component of the vitreous humour in the eye and forms the basis for articular cartilage and hyaline cartilage. Moreover, collagen type II makes up 50% of all protein in cartilage and 85-90% of collagen of articular cartilage. As with collagen type I, the fibrils in collagen type II are also orientated randomly in the viscous proteoglycan matrix. Both collagen type I and II macromolecules impart a strength and compressibility to the matrix and allow it to resist large deformations in shape due to joint mobility. This property allows joints, for example, to absorb shock \cite{23}.

1.2.5 Collagen Extraction and Immunogenicity

1.2.5.1 Extraction

Collagen can be extracted from various tissues in animals. Biological tissues consist of monomeric (acid soluble) and polymeric (insoluble/ acid stable). Only monomeric collagen can be extracted and represents just about 10% of the collagen in tissues. In mature tissues, such as tendons, the bulk of the collagen consists of the polymeric version which is insoluble in acids since it is highly cross-linked. As described earlier, monomeric collagen slowly turns into the polymeric version as the natural cross-links are chemically rearranged with age to form acid-stable aldmine cross-links. Younger animals are used to extract collagen as the amount crosslinking increases with age (e.g. 2 year old bovine) \cite{12}.

The process of collagen extraction utilises weak acid solutions and a series of steps which can be divided into different approaches: acidic or enzymatic (pepsin). The extracted collagen solubilised in acid at pH 2 (0.01 M
HCl or 0.1 M acetic acid) and is either named pepsin soluble collagen (atelocollagen) or acid soluble collagen (telocollagen) depending on the extraction method. Acid extraction process results in the preservation of the telopeptides. Pepsin extraction results in the removal of most the telopeptides resulting in atelocollagen.\textsuperscript{[24]} The non-helical telopeptides on the C- and N-terminals in native collagen aid in collagen fibril alignment but are not required for fibrillogenesis. Extracted fibrillar collagens (e.g. I, II, III, and V) can form fibrils in a 64-67-nm staggered manner \textit{in-vitro}. The extracted collagen solutions are solubilised in acid assemble into fibrils upon warming and pH neutralisation, they form a hydrogel with a mesh network of fibrils. Collagen fibrils self-assemble at neutral pH into bundled fibres which crosslink forming a matrix structure that forms a hydrogel\textsuperscript{[25]}. A hydrogel is a 3D polymer network and in healthcare hydrogels often involve water as the interacting solvent\textsuperscript{[26]}. The speed of fibril formation, the fibril thickness, and the fibril length are easily affected by pH, the concentration of sodium chloride (NaCl), and temperature \textsuperscript{[27]}.

\textbf{1.2.5.2 Immunogenicity}

The term immunogenicity refers to the ability of a substance to induce a cellular and humoral immune response, while antigenicity is the ability to be specifically recognised by antibodies that are generated as a result of the immune response to a given substance\textsuperscript{[28]}. The triple helical region of collagen has very little variations in the amino acid sequence (only a few percent) between mammalian species due to a high degree of evolutionary stability (sequence homology). There is a far greater degree of variation in the telopeptide (non-helical) regions, where up to half of the amino acid residues exhibit interspecies variation. The major antigenic determinants are located within the telopeptide regions. Some studies have shown that the helical regions also play a major role in collagen–antibody interactions. Although much less likely to occur, these interactions come into play especially when the collagen is degraded by collagenases exposing those regions to the immune system\textsuperscript{[28],[29]}.\textsuperscript{[29]}.
In general, macromolecular features of a protein not common to the host species are more likely to encourage an immune response than shared features. The purity of the collagen preparation also plays a role in the risk of the immunogenicity. Most clinically used collagen utilises atelocollagen which is mostly devoid from the non-helical regions. The collagen extraction process (Figure 7) involves the use of pepsin does not completely remove all the non-helical peptides. As a result, there is still a small risk that those regions can be recognised by the immune system [29]. Despite this, collagen is widely considered a weak antigen as a very small percentage of people possess humoral immunity to collagen. A simple serological test can be used to determine if a patient will likely be susceptible to collagen reactivity [30].

Figure 7. Atelocollagen production by the cleavage of telopeptides using pepsin, Reproduced from Lynn, Yannas, and Bonfield 2004 [29].

As there is a small risk of immunogenicity, collagen products have been widely used in the clinic for decades, with an excellent safety profile. For example, two commercial products for dermal use are Zyderm® and Zyplast® collagen implants, which were commercially launched in the USA in the 1980s for use in soft tissue contour irregularities. Since that time more than 750,000 patients have used these products. The products contain purified bovine collagen which is comprised of 95 % collagen type I and 5% collagen type III.
The most prevalent adverse reaction is localized hypersensitivity at treatment sites, occurring in 3% of skin tested patients and 1-2% of the treated patients. Circulating antibodies to bovine collagen can be demonstrated in the sera of patients with local hypersensitivity (90–100%) [31].

There is potential to almost eliminate immunogenicity when human collagen can be produced in a large scale by heterologous expression in mammalian, insect, and yeast cell lines. Collagen has been produced in Escherichia coli with the human sequence but not at a large scale [5]. Human transgenic collagen type I has also been prepared from transgenic tobacco plants [32].

1.3 Collagen Function in the Eye

The anatomy of the eye (Error! Reference source not found.) can be broadly divided into two segments; the anterior segment and the posterior segment. The anterior segment consists of the cornea, conjunctiva, aqueous humour, iris, and ciliary body. The posterior segment constitutes of the sclera, choroid, Bruch’s membrane, retinal pigment epithelium (RPE), neural retina, vitreous humour and the optic nerve [33].

1.3.1 Anterior Segment of the Eye

The cornea is thin, transparent, smooth, avascular, highly innervated and the most sensitive tissue in the body. It is convex, aspherical in shape and directly exposed to the external environment and is suffused by the tear film and its inner surface is directly in contact with fluid called the aqueous humour. The thickness of the cornea gradually increases from the centre to the periphery. The corneal stroma is embedded with a relatively homogeneous and uniform arrangement of collagen type I fibres (diameter 25–35 nm). In the cornea, there are about 70 microfibrils within each fibril which are tilted by about 15° to the fibril long axis. This tilt reduces the usual 67 nm axial periodicity seen in tendon and sclera, to closer to 65 nm. This alignment of collagen fibrils allows the cornea to transmit over 90% of the incident light at visible wavelength [34], [35]. Such arrangement is thought to prevent scattered light interference on the cornea caused from the incident light. The arrangement and function of the
collagen fibres allow the cornea to remain transparent so that the light rays can pass into the eye. Corneal smoothness is maintained by the corneal epithelium and tear film [33].

Collagen type I represents the vast majority (90%) of collagen in the human body [36]. In the cornea and sclera, type I collagen represents the largest proportion of collagen (~70%), along with minor amounts of different collagen types including type III, V and VI. The coexistence of different collagens aids in fibril arrangement and the final structure of the cornea and the sclera. In the cornea, collagen type I fibrils confer clarity as well as mechanical stability, due to a uniform fibril diameter (~25 nm) which is narrower than in many other connective tissues. In the sclera collagen type I also helps resist the tensile forces due to the intraocular pressure (IOP) to maintain the structure of the ocular globe and to protect the inner ocular tissues.

The structural arrangement of collagen type I in the sclera is opaque and is composed of collagen fibrils with various diameters ranging from 25-230 nm. The collagen fibrils form bundles, which are not parallel but are entangled in individual bundles. The reasons why the cornea is transparent (and the sclera opaque) are not fully understood [37], [38].

The aqueous humour is an optically clear, slightly alkaline ocular fluid that is continuously formed (~2.5 μL/min) from plasma by epithelial cells of the ciliary body. It is estimated that the entire aqueous humour in the anterior cavity of the eye is replaced approximately every 100 min. The formation of the aqueous humour is mainly controlled by the interaction of inhibiting α2-adrenoreceptors and stimulating β-adrenoceptors. The aqueous humour supplies nutrients and oxygen to the avascular ocular tissue of the cornea and lens. The IOP in the eye is controlled by the rate of aqueous humour production and the resistance to its outflow [39], [40].

### 1.3.2 The Vitreous Humour

The posterior cavity of the eye is transparent and contains a thick, gel-like fluid which covers the space between lens and retina. It is called the vitreous humour (VH) or vitreous body and aids in maintaining the structure of the globe of the eye and represents about 80% of the eyeball volume (Error! Reference source not found.) [41].
The physical characteristics of the VH (Table 2) enable it to carry out its role in maintaining clear vision as well other biological functions, which include oxygen and nutrients, as well as drug diffusion inside the back of the eye [42].

**Table 2. The physical characteristics of the vitreous humour. Adapted from Donati et al 2014 [42].**

<table>
<thead>
<tr>
<th>Physical Characteristics of the Vitreous Humour</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight</td>
<td>4g</td>
</tr>
<tr>
<td>Density</td>
<td>1.0053-1.0008 g/cm³</td>
</tr>
<tr>
<td>Refractive index</td>
<td>1.3345–1.3348</td>
</tr>
<tr>
<td>Viscosity</td>
<td>0.3-2 Pas</td>
</tr>
<tr>
<td>pH</td>
<td>7.0-7.4</td>
</tr>
</tbody>
</table>

In humans, the VH is composed of 98-99% water with the remaining components being mainly collagen type II (40-120 μg/mL) and hyaluronic acid (HA, 100-400 μg/mL), as well collagen type IX which largely contribute to the physical and mechanical properties of the vitreous. About 15-20% of water in the VH is present in bound form to e.g. HA, which further contribute to viscosity of the vitreal structure [42], [43]. Variations in collagen and HA concentration occur largely due to age where they generally decrease with age, with collagen thought to form insoluble fibrils with in the VH. Other VH components include soluble proteins (200-1400 μg/mL), albumin and iron-binding proteins which represent the largest portion, 40% and 30% respectively [43].

The VH also contains a small number of cells such as fibroblasts and hyalocytes which are responsible for vitreous matrix creation and maintenance such as the production of collagen and HA. Different types of collagen exist in the vitreous, however, collagen type II is the main component (~70%). Other minor components of the VH contribute to its viscosity and ability to provide mechanical support for the eye.
1.3.3 Hyaluronic Acid (HA)

HA is largely responsible for the mechanical properties of the vitreous. The HA chemical structure (as seen in Figure 8) is made of 1,4-linked D-glucuronic acid and D-N-acetylglucosamine. HA is a major component of the ECM and belongs to a group of macromolecules called glycosaminoglycans (GAGs) which provide a large variety of function including bridging and linking adjacent collagen fibrils in the cornea. GAGs are unbranched polysaccharide chains composed of repeating disaccharide units. [44].

![Figure 8](image)

Figure 8. The chemical structure of the hyaluronic acid (HA) polymer composed of D-glucuronic acid and N-acetyl-D-glucosamine linked by glycosidic bonds.

HA synthesis is achieved through the enzymatic activity of hyaluronansynthases acting at the internal face of the plasmatic membrane and extruding the nascent polymer to the extracellular medium. HA can entrap large amounts of water and ions to provide tissues with hydration. In fact, HA can absorb a large number of water molecules due to hydrogen bonding. This allows to form a gel in the vitreous at very concentrations. HA is metabolically very active in the body, for example, its half-life in the skin is less than one day [45], [46].

The molecular weight of HA varies depending on the tissue, for example, it is approximately 5 MDa in the vitreous humour and 2 MDa in the ECM [47], [48]. This therefore affects the rheological properties of HA in different tissues as the viscosity of a polymer depends on and is directly related to the molecular weight of the polymer [49].
1.3.4 Artificial Vitreous Humour Substitutes

Collagen type II fibrils are separated by the large HA molecules which prevent any further polymerisation (crosslinking) of the fibrils. With age break down of the HA results in reduction in viscosity/liquefaction of the VH. This in turn allows collagen fibrils to crosslink and form larger fibres which distorts the vitreous structure further. As Liquefaction occurs, the anterior aqueous humour may permeate into the posterior vitreous, resulting in a tugging effect at the attachment point of retina and vitreous fluid. The release of cells into the fluid, which appear as floaters, and if a significant tugging effect is developed it may pull away or detach the retina. Diseases affecting the posterior segment of the eye are difficult to reach and treat and can result in defects in the VH. Age-related macular degeneration (AMD), macular oedema, proliferative diabetic retinopathy and glaucoma are some of the common posterior eye diseases that may also lead to vision loss if not treated.

Such pathologies or just old age often require partial or total vitreous removal. Presently, temporary and permanent intraocular vitreal substitutes mainly have a structural function to ensure retinal adherence following cryo- or laser retinopexy for the necessary time, to control intraocular haemorrhages, and to maintain the IOP. The incidence of retinal detachment requiring vitreous replacement (or some form of internal tamponading) is 6.3-17.9 per 100000 population. There are 100 million patients with diabetic retinopathy, and many may eventually require some form of internal tamponading (e.g. silicone oil). The current options often lead to complications including the development of nuclear cataract, glaucoma, corneal decompensation, and retinal re-detachment. Considerable research has been conducted to produce a vitreous substitute that maintains the IOP and with viscoelastic characteristics that reduce the shear stress on the retina.

Many approaches to replace parts of the vitreous have been evaluated over the years and have included gases, liquids (e.g. saline, semifluorinated alkanes, silicone oils, heavy silicones, magnetic silicones). Inert and colourless gases (mainly N₂, O₂ and CO₂) are commonly used during the surgical procedures. These gases however, diffuse easily in the blood circulation, reducing the tamponade effects in a few days. Some of the other...
investigated gases include sulphur hexafluoride (SF₆), perfluoroethane (C₂F₆), and perfluoropropane (C₃F₈) which also are colourless, odourless, inert, nontoxic, and expansive gases. They present a high surface tension and have a specific gravity lower than water to maintain the tamponade effect [⁵¹]. For example, an inert gas such as SF₆ will expand when injected into the eye due its lower water solubility. The surface tension then prevents any fluid movement into the retinal breaks. This results in the support of the physiological fluid removal from the retinal space which then allows chorioretinal adhesions resulting in retinal tamponading [⁵³], [⁵⁴].

Current vitreous substitutes present issues which include that patients with intraocular gases are advised against air travel or traveling to high altitude, since the reduction of atmospheric pressure leads to expansion of intraocular gas bubble and cause considerable increase of IOP. Diving must be avoided as the hyperbaric pressure occurring during scuba diving causes hypotony and partial globe collapse [⁴²]. An artificial replacement needs to interact with the biology and metabolism of the eye to permit the transportation of substances, ions, and oxygen and to maintain integrity and transparency over time. The ideal properties of an artificial vitreous substitute are summarised in Table 3.

Table 3. The characteristics of an ideal vitreous humour. Adapted from Soman and Banerjee 2003 [⁵²].

<table>
<thead>
<tr>
<th>The ideal vitreous substitute</th>
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</thead>
<tbody>
<tr>
<td>Mimic the native vitreous</td>
</tr>
<tr>
<td>Have similar viscoelastic proprieties to the native VH</td>
</tr>
<tr>
<td>Clear and transparent</td>
</tr>
<tr>
<td>Have refractive index and density similar to that of the native vitreous</td>
</tr>
<tr>
<td>Biocompatible and chemically inert</td>
</tr>
<tr>
<td>Maintains the IOP within a physiologic range and support the intraocular tissues in position</td>
</tr>
<tr>
<td>Allows movement of ions and electrolytes</td>
</tr>
<tr>
<td>Easily available, stable, and injectable through a small syringe</td>
</tr>
<tr>
<td>Can maintain its light transparency after surgery without undergoing opacification</td>
</tr>
</tbody>
</table>
Vitreous substitutes are also required for the pharmaceutical development of new intraocular medicines. The global number of intravitreal injections clinically has grown exponentially during the last 10-15 years with the introduction of antibody-based medicines to treat wet age-related macular degeneration (AMD). The number of intravitreal anti-VEGF injections has in fact reached almost 20 million per year globally [55]. Wet AMD is the main cause of blindness in the elderly and until the introduction of ranibizumab (antibody fragment) and aflibercept (Fc-fusion) treatment was not possible or reliable. With the ageing population, there is a continued need for intraocular medicines to treat a wide range of blinding conditions (e.g. diabetic macular oedema and inflammation caused by uveitis) and to develop medicines that have extended duration of action. Current AMD treatments require administration about once every 2 months. Intravitreal injections can be painful and are understandably difficult for patients, so there is global effort to develop strategies for longer acting intravitreal medicines.

There are two types of AMD which are termed as wet and dry AMD. Dry AMD represents about 90% of all cases in which layers of the macula get progressively thinner and atrophy. Dry AMD is a chronic condition which causes a degree of visual impairment which can progress to blindness. Wet AMD (also called neovascular AMD) is a condition in which new blood vessels grow in the choroid layer and are weak, leak fluid, lipids, and blood. Wet AMD can rapidly cause blindness if left untreated [56].

Pharmaceutical preclinical models are widely used to develop dosage forms for all modes of administration (e.g. oral, pulmonary, topical). For example, drug dissolution rates are widely studied in laboratory models to optimise oral dosage forms prior to evaluation in animal. With the advent of intraocular medicines has come the development of a 2-compartment outflow model of the eye designed to estimate human clearance times of preclinical candidates [57]. As with preclinical models used for developing oral dosage forms, models for developing intraocular dosage forms require simulated biological fluids. In the case of the eye, there is a need for simulated vitreous fluid.
1.3.4.1 Hydrogel-Based Vitreous Substitutes

A variety of hydrogels have been tested for use as an artificial VH, including polyvinyl alcohol, poly(2-hydroxyethyl methacrylate), hydroxypropyl methylcellulose and polyacrylamides. Unfortunately, they also presented issues such as vitreous opacification, short residence time, immune reactions, toxicity or poor tamponade effect [42], [51].

A recent strategy to make a vitreous substitute was to prepare a thermosetting gel composed of a mixture of methylcellulose and poly(ethylene glycol). Gelling occurred at 32-34°C and showed a promising outcome in-vivo, including retaining transparency after injection and a good tamponade effect [58].

Hyaluronic acid (HA) can provide a good tamponade effect (due to its high hydrophilicity) and excellent biocompatibility. HA is degradable which is a limitation for its clinical use. Chemical crosslinking of HA is used to improve HA duration in the body for various biomedical applications. HA products for tissue augmentation include chemically HA crosslinked with 1,4-butandiol diglycidylether [59]. Recently, a HA based vitreous substitute evaluated preclinically was prepared by using a thiol-modified HA which becomes crosslinked through air oxidation of thiols into disulfides [60], [61]. The number of unreactive thiols remaining is an issue as free thiols can undergo reaction with endogenous proteins [62]. Shafaie et al interestingly reported that 4.5 mg/mL HA (Mw: 53.2 KDa) matched the viscosity of the human vitreous at 1 Hz which is an example that could be explored further [63].

Photochemical crosslinking is used in the clinic to crosslink collagen type I in the cornea for keratoconus [64], [65]. Riboflavin is used as a photoinitiator and is applied as a solution corneal surface. Upon exposure to blue light, riboflavin creates a reactive oxygen species (ROS) which cause collagen crosslinking to increase the mechanical strength of the cornea. The crosslinking process occurs between the amino acids histidine, hydroxyproline, hydroxylysine, tyrosine, and threonine which are involved in the crosslinking process [66]. The reaction can potentially be used to prepare a vitreous substitute. HA derivatised with the amino acid tyramine (HA-T) can also be photochemically crosslinked which utilises the phenol groups in
tyramine \textsuperscript{[67, 68]}. This indicates that HA may be photochemically crosslinked with collagen \textit{in-situ} to prepare an artificial vitreous substitute.

\textbf{1.4 Collagen Based Biomaterials}

\textbf{1.4.1 Biomedical Applications of Collagen}

The most commonly used sources of collagen for biomedical applications are from bovine skin and tendons; porcine skin, intestine, or bladder mucosa; and rat tail. The fibril-forming collagens represent the most commonly used forms of collagen-based biomaterials. Collagen type I is by far the most abundant in animals and is therefore the most commonly used. Properties of collagen-based biomaterials are influenced by the source of collagen as well as by the method of collagen extraction including purification, fibril formation, casting and subsequent crosslinking. For cosmetic applications where collagen does not get absorbed, and hence amino acid differences play less of a role, the main source of collagen is from fish skin due to low cost \textsuperscript{[5, 69]}. Exogenous collagen benefits from enzymatic degradability by endogenous collagenase, gelatinases and telopeptide-cleaving enzymes \textsuperscript{[70]}. Collagen types I–III are hydrolysed by matrix metalloproteinases (MMP) -1, MMP-2, MMP-8, MMP-13, and MMP-14, which belong to the collagenase class of enzymes. The resulting collagen fragments are then degraded further by gelatinases and nonspecific proteases. For example, MMP-2 breaks down bovine type I collagen \textsuperscript{[71]}. In fibril form, the peptide bonds in the collagen triple helix are occluded from the active sites of enzymes. Telopeptides and non-fibrillated triple helices are cleaved into single-stranded regions by MMPs (e.g. MMP-4). Then gelatinases like MMP-2 cleave the triple helices. Chemically crosslinked collagen is therefore more difficult to degrade. When exogenous collagen degrades it can elicit a complex response which can be beneficial for the host \textsuperscript{[72, 73]}. Some degradation products of collagen types I–III induce chemotaxis of human fibroblasts which promote restoration of tissue structure and functionality \textsuperscript{[5]}. Collagen has an affinity for biomolecules and participates in organisational and macromolecular arrangement that resembles the ECM. Many cell-surface proteins bind to collagen where several different cell surface
receptors recognise collagenous, triple-helical sequences to mediate cell adhesion to collagens. Cell–collagen interactions are mediated by four different groups of receptors. Firstly, glycoprotein VI receptors (platelet collagen receptors) recognise peptide sequences containing the Pro-Hyp-Gly unit. The second group belongs to receptors of the integrin family and discoidin domain receptor 1 and 2, which bind to the Phe-Hyp-Gly sequence of collagen. Receptors of the integrin-type are the third receptor group and they recognise cryptic motifs within the collagen. The final group of receptors are those with the affinity for non-collagenous domains. Many proteins such as decorin and laminin that contain integrin-recognition sequences can bind to both collagen and integrins, promoting cell adhesion and proliferation. This allows collagen biomaterials interact well with cells well and integrate into biological tissues to an excellent degree [2], [5].
1.4.2 Collagen-Based Systems for Tissue Regeneration

Collagen can be prepared into cross-linked compacted solids or into lattice-like gels. The main clinical applications of collagen are for burn/wound dressings, osteogenic and bone filling materials, anti-thrombogenic surfaces and immobilisation of therapeutic enzymes [69], [74].

In research, collagen gels for tissue engineering have also been prepared by the Real Architecture For 3D Tissues (RAFT) technology. Collagen gels are first prepared and then a large amount of the gel water content is removed using a highly absorbent membrane resulting in a stable collagen system that can be utilised in various tissue engineering applications [75].

One of the most commercially profitable uses of collagen is for the intradermal injection of soluble collagen to modify dermatological defects (tissue augmentation), including wrinkles, nasolabial folds and to aid with the healing of acne scars. Commercial products include Zyplast® (telocollagen) and Koken® (atelocollagen) which are both bovine derived [69].

Collagen-based conduits (e.g. NeuraGen®) are used clinically for the treatment of peripheral nerve injury. Collagen is ideal for such application as it constitutes nearly 50% of the protein content in the peripheral nerve, is well integrated into tissues, allows for excellent cell attachment alignment and has a slow and predictable biodegradability [76], [77].

Resorbable forms (not chemically crosslinked) of collagen have been used to dress oral wounds and for closure of graft. Collagen-based membranes have also been used in periodontal and implant therapy (e.g. Mucoderm®) [78] as barriers to prevent epithelial migration and allow cells with regenerative capacity to repopulate the defect area. It has been suggested that membrane regenerative techniques facilitate the natural biological potential by creating a favourable environment for periodontal and peri-implant regeneration [79].
1.4.3 Collagen-Based drug Delivery Systems

Numerous collagen-based drug delivery systems are used clinically with films being a very a common example. Experimentally, collagen films have been prepared from reconstituted atelocollagen incorporated with drugs such as tetracycline. Films are formed by air-drying a casted collagen preparation into which drugs can be loaded by hydrogen bonding, covalent bonding or simple entrapment \[^{[69]}\]. Collagen films can be used for the treatment of tissue infection, such as infected corneal tissue \[^{[70],[79]}\].

Clinically, collagen shield inserts like Ocusert have been used to deliver pilocarpine over 7 days for the treatment of glaucoma. The insert is no longer routinely used due to side effects such poor insert retention in the target area (under eyelid) and blurred vision \[^{[80]}\]. One of the major applications of collagen is wound dressings (e.g. DermaCol/Ag™) to promote wound healing and deliver antimicrobial agents. DermaCol/Ag™ is used for wound healing and infections by delivering silver ions \[^{[5],[81]}\]. Apligraf® is approved for ulcer wound treatment and is composed of collagen gel-populated fibroblast overlaid by an epidermis \[^{[74]}\].

Gelatine is derived from denatured collagen and is also used in many biomedical applications. During heating, the triple helix of collagen unwinds, and the chains separate. As the denatured mass of tangled chains cool, they absorb water. The denatured product is the gelatine and is widely used in both cosmetic and biomedical applications such as capsule shells in medicines \[^{[70]}\].

1.4.4 Challenges and Solutions to Collagen Biomaterials Applications

Collagen in its native environment in tissues has proteolytic resistance and high tensile strength due to a high number of covalent cross-links formed post fibril assembly (non- and enzymatic crosslinks). Upon extraction the collagen solution forms a hydrogel due to fibril assembly upon neutralisation and heating. The hydrogel is mechanically weak due to the lack of covalent crosslinks that strengthen to the fibrils \textit{in-vivo}. The hydrogel contains large amounts of water and lacks many of native covalent crosslinks and is mechanically weak. Issues such as disintegration on handling or under the pressure of surrounding tissues \textit{in-vivo} commonly occur \[^{[27]}\]. The advantages
and disadvantages of using collagen as a biomaterial are also summarised in Table 4 (next page).

Efforts have been made to control the rate of degradation and in-vivo absorption of collagen by generating new cross-links. Typically, in-vitro cross-linking of collagen enlists its amino and carboxyl groups to form new covalent bonds. Approaches have included crosslinking collagen with various reagents including formaldehyde, glutaraldehyde, carbodiimides, polyepoxy compounds, acylazides, and hexamethylene diisocyanate. Cytotoxicity is a major concern due to difficulties to remove the toxic reagent completely and the residual electrophilic reagents and compounds formed during degradation \[5\]. The carbodiimide crosslinking reaction that is commonly used to crosslink collagen-based materials to enhance their mechanical strength \[82\]–\[84\]. Photochemical crosslinking is used to increase the mechanical strength of collagen assemblies. Such crosslinking methods turn collagen into a new entity which has to undergo new testing for biocompatibility and biodegradability \[85\].

Transglutaminase catalyses an acyl-transfer reaction between the g-carboxamide groups of peptide-bound glutamine residues and the 3-amino groups of peptide-bound lysine residues, resulting in g-glutamyl-3-lysine isopeptide bonds. This method not only makes collagen more protease resistant but also increases its mechanical stiffness while generating no cytotoxic by-products \[86\]. Fortunati et al has shown very promising data with regards to crosslinking collagen type I using tissue transglutaminase for bone healing indicating a potentially clinically applicable method to crosslink collagen \[87\].
Table 4. The advantages and disadvantages of using collagen-based biomaterials. Reproduced from Lee, Singla, and Lee 2001 [70].

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Available in abundance and easily purified from living organisms</td>
<td>High cost of pure type I collagen</td>
</tr>
<tr>
<td>Biodegradable and biocompatible</td>
<td>Small risk of antigenicity</td>
</tr>
<tr>
<td>Biological plastic due to high tensile strength and minimal expressability</td>
<td>Variability of isolated collagen (e.g. crosslink density, fibre size, trace impurities such as collagen type II)</td>
</tr>
<tr>
<td>Hemostatic: promotes blood coagulation</td>
<td>Hydrophilicity which leads to swelling and more rapid release;</td>
</tr>
<tr>
<td>Formulated into different forms (e.g. films, sheets, sponges and beads)</td>
<td>Variability in enzymatic degradation rate as compared with hydrolytic degradation</td>
</tr>
<tr>
<td>Biodegradability can be regulated by cross-linking</td>
<td>Complex handling properties</td>
</tr>
<tr>
<td>Easily modifiable by utilizing its functional groups</td>
<td>Poor mechanical properties</td>
</tr>
<tr>
<td>Compatible with synthetic polymers</td>
<td></td>
</tr>
</tbody>
</table>

1.4.5 Hydrogels for the Delivery of Therapeutics

The delivery of drugs and cellular therapies can play a crucial role in the treatment of diseases. Drug and cell therapies can be delivered into the body through various routes e.g. subcutaneously, orally, and parenterally. Depending on the therapeutic agent challenges arise with low level of macromolecular absorption, slow onset of action, drug degradation and cell survival (in cell therapy) [88], [89]. To address the issues, research has focused on delivery systems that achieve a more localised delivery of therapeutics [90]. Localised delivery systems limit the systemic drug exposure and maximises its release at the site of action, and potentially reduces dosing frequency, [89], [91]. An effective delivery system (implant) needs to provide drug retention by a delivery vehicle, allowing for targeted delivery and release. Such systems can minimise the fluctuations in drug levels by providing a constant supply of drug at the site of action (e.g. Ocusert). Biodegradability is important to negate the
need for surgical removal such as in the case of contraceptive implants. Solid implants can present a risk of scar tissue capsule formation around the implant due to the immune response [88].

Hydrogels are particularly appealing systems for use in drug delivery, regenerative medicine and diagnostics experimentally [92]. The high-water content (~70–99%) in most biomedical hydrogels can provide them with a physical similarity to the ECM [90]. Hydrogels can be composed of networks derived from molecular entanglements (non-covalent interactions) such as ionic, H-bonding or hydrophobic interactions, and of course, covalent crosslinks [93].

Depending on the cross-link density, a mesh of entangled chain can form within a hydrogel to slow liquid and small solute diffusion. The mesh size can be varied by controlling the density of crosslinks. The diffusion and release rate (Figure 9) of therapeutic agents can depend on the mesh size and whether there is any non-covalent association of the drug with the hydrogel. When a therapeutic agent is larger than the mesh size, assuming it has been or can be mixed in the gel, the therapeutic agent can become entrapped within the gel and is released when the gel degrades, swells or deforms [90], [94].

Hydrogels have the potential to preserve the stability of therapeutic proteins due to hydration and limited protein mobility in the hydrogel network. The protein may be released in a controlled fashion and maintain a therapeutic concentration in the surrounding tissues or in the circulation [95]. Lima et al prepared a non-toxic pH-responsive alginate-based hydrogel for protein delivery which showed a pH dependent release and kept bovine serum albumin (BSA) stable at pH 1.2, in-vitro. Albumin is not a very therapeutically relevant protein [95], but is often used as a model protein as it is economically available.
Cell-based therapies are a promising tool to replace abnormal or injured tissues (such as neurons and bones) and to treat diseases including cancer. Alofisel is solution of allogeneic (donor-derived) expanded adipose-derived stem cells (eASCs), which show immunomodulatory and anti-inflammatory effects at inflammation sites [96]. Cell survival and attachment at the target site are still issues that limit the success rate of the treatment [97]. One approach involves encapsulating cells by entrapment within a semi-permeable membrane. Such a system does not provide a proper in-vivo biomimetic microenvironment for cells, which can result in poor control of cell proliferation, migration, differentiation [93].

Hydrogels have attracted major research interest for use as scaffolds for regenerative medicines as they can be designed to contain pores large enough to accommodate living cells. Cell behaviour and signalling is influenced by the mechanical properties of the environment where the cells

Figure 9. Mesh size mediates drug diffusion. A small therapeutic agent relative to the mesh size diffuses rapidly through the hydrogel. Release slows down when the size of the agent is close to the mesh size. When the drug is larger than the mesh size, agents are entrapped inside the network and are released upon gel degradation or swelling. Adapted from Li, J. & Mooney, D. J. 2016 [90].
grow. On stiffer substrates cells have stiffer, more organised cytoskeletons and more stable cell-surface adhesion points. Cells such as fibroblasts can sense the mechanical stiffness of the ECM and other cells. In a stiff matrix fibroblasts are proliferative, fibrogenic and produce stress fibres \cite{98}. Hydrogels with tuneable mechanical properties can therefore provide a more biomimetic environment for cells \cite{98}, \cite{99}. A low mechanical strength and difficulties in handling are major hurdles to overcome for many hydrogels. Mixing of therapeutics with hydrogels is a major issue as damage to the hydrogel structure occurs \cite{93}.

A more ideal type of hydrogel for the delivery of therapeutic agents is the in-situ gelling type. An in-situ gelling hydrogel is delivered to the body as a solution which then forms a gel in response to various stimuli such as pH, temperature ad light. The solution can be formulated with drugs or cells and then injected into the target area where it subsequently forms a gel depending on the mechanism. The release of toxic by-products, toxic crosslinkers, biodegradability and biocompatibility are issues of encounter with in-situ hydrogels \cite{100}.

Stimuli responsive polymers are a class of polymers which can undergo physical or chemical changes in response to external changes such as pH, ionic factors, chemical agents, and mechanical stress. Poly(N-isopropylacrylamide) (PNIPAAM) is a temperature sensitive polymer which forms a gel by undergoing a reversible volume phase transition caused by the coil-to-globule transition at a specific temperature. The polymer intramolecular collapse occurs before intermolecular aggregation above the lower critical solution temperature (LCST) and the collapse of individual polymer chains increases the scattering of light in solution (cloud point). PNIPAAM and its derivatives have been investigated for drug delivery. The high burst release and long term biocompatibility are issues that remain to be overcome \cite{101}, \cite{102}.

Qiu et al prepared an in-situ forming hydrogel by functionalising poly(ethylene glycol) (PEG) with thiol groups through disulfide formation. The functionalised PEG molecules were mixed with a crosslinker (α, ω-divinylsufone-poly(ethylene glycol)) and BSA as a model protein. BSA release occurred over 25 days with little observed burst release. However, the toxicity of the crosslinker and unreactive thiols can be an issue. Gelling starts as soon
as the polymer the crosslinker are mixed which mean injectability can be an issue \[103\].

In a recent study conducted by Gau et al they successfully prepared an injectable self-healing conductive hydrogel based on N-carboxyethyl chitosan and dextran-graft-aniline oligomers which was biodegradable and biocompatible. It was used as a cell delivery carrier for myoblast cell therapy to enhance skeletal tissue repair and showed very promising results when injected in a rat model of volumetric muscle loss injury of the skeletal muscle. The release of the cells is not predictable and no long term toxicity study on the effect of the biomaterial on the heart was conducted \[104\].

Recently, a biocompatible and biodegradable in-situ polymerisable collagen (IPC) solution which forms a collagen hydrogel upon contact with biologically relevant media was prepared. The IPC uses sodium ethylenediaminetetraacetic acid (EDTA) to stabilise the collagen fibrils and keep them soluble at pH 7 \[105\]-\[107\]. After injection upon contact with biological media the EDTA molecules diffuse from the collagen triggering fibrillogenesis (gelling) \[106\]. An IPC was successfully evaluated in phase I and II clinical trials for tissue augmentation and was well tolerated and none of the participating subjects displayed signs of hypersensitivity \[108\], \[109\]. EDTA is safe and is present in many clinically approved formulations such as DermaCol/Ag\textsuperscript{TM} \[110\] and is given parentally for lead poisoning \[111\]. The IPC solution may be formulated with therapeutics and delivered using a small gauge needle. The IPC process does not involve modifying the collagen by chemical crosslinking and allows for in-situ gelling without the release of toxic by-products \[105\]. An IPC solution is potentially versatile and might allow the preparation of a gel with tuneable mechanical properties by increasing in fibril density which reduces the mesh size. A hydrogel like IPC can potentially be an excellent candidate to act as a vehicle for cellular and protein therapeutics. The collagen in IPC can adhere to tissues and cells inside the gel can also attach and proliferate.
1.5 Ocular Drug Delivery

The development of a slow release ocular drug delivery system is challenging for both the posterior and anterior segments of the eye. Ocular tolerability is a key challenge. Blinding conditions that affect the posterior segment require slow release formulations to minimise the frequency of dosing which is by intravitreal injection. Intravitreal injections are exceedingly invasive, and much research is being conducted globally to increase the duration of action of intraocular drugs so that the frequency of injection can be reduced. The need for ocular tolerability poses significant challenges when it comes to formulating slow release formulations including volume, presence of particulates, and effects on IOP, sight and the immune system[^112].

Therapies that target conditions of the anterior segment such as infections and scaring are topically instilled. Topically applied drugs must first permeate through the cornea to reach the aqueous humour to distribute to surrounding tissue. Unfortunately, most preferable routes of absorption for eye drops is the conjunctiva, nasal ducts and the cheeks. A topical ocular formulation is normally eliminated quickly which is insufficient, and the tissues need to be exposed to the drug for a prolonged period to causes a therapeutic effect[^113].

There is a lot interest in developing ocular drug delivery systems which require less a frequent application. There is also a growing interest in cell therapies to treat conditions which can cause blindness. Hydrogels may be an excellent vehicle to deliver cells into the eye as they allow for cell distribution, survival, and integration in the host tissue[^114],[^115]. Hydrogels can protect the proteins and small molecule drugs from certain degradation pathways and protect transplanted cells from rejection by the immune system[^116],[^117].
1.5.1 Trachoma

Trachoma is the most common cause of infectious blindness worldwide and is caused by a bacterial infection from *chlamydia trachomatis* [118]. Active trachoma affects 21 million people of which visual impairment occurs in about 2.2 million people worldwide of which about 1.2 million are completely blind [118], [119]. The infection is spread by direct contact through infected nasal or ocular secretions on fingers or clothes, as well as eye-seeking flies [120]. The infective pathogen triggers a chronic inflammatory response in the conjunctiva which is termed as an active trachoma.

Trachoma is clinically characterised by follicles and papillae (trachomatous inflammation-follicular). The follicles are composed of subepithelial collections of lymphoid cells which appear as small, yellow-white elevations, whereas the papillae are engorgement of small vessels with inflammatory conjunctival thickening. Then after repeated episodes of infection and inflammation, later in life, scar tissue begins to accumulate within the conjunctiva. This particularly affects the conjunctiva in the lining of the upper eyelid which causes in-turning of the eyelid (entropion) which leads to the rubbing of lashes on the cornea (trachomatous trichiasis). Ultimately, without treatment sight is lost due to an irreversible blinding corneal opacification. Active trachoma is most common in children younger than 5 years and the prevalence can reach 60% or more. In endemic areas, the disease process starts in early childhood with recurrent conjunctival infection by the bacterium [121].

Oral treatment with the macrolide antibiotic azithromycin has been shown to reduce the occurrence of trachoma after multiple doses [122]. The world health organisation (WHO)-led Global Alliance for the Elimination of Trachoma by 2020 (GET 2020) introduced the SAFE strategy. This strategy involves surgery for trichiasis, mass distributing antibiotics to treat the infection, promoting facial cleanliness to prevent disease transmission, and increasing access to water and sanitation (environmental improvement). The drug treatment recommends the administration of oral azithromycin to children (1-9 years old) in any areas where the prevalence of trachomatous inflammation follicular is 10% or greater [120].
1.5.1.1 Treatment of Trachomatous Trichiasis

Trachomatous trichiasis can lead to lashes chafing against the ocular surface, resulting in irritation and inflammation. Corneal abrasion may occur after prolonged or severe exposure, causing more discomfort and potentially keratitis (corneal inflammation). Corneal scarring can then occur if this condition is not properly managed \(^\text{[123]}\). In milder cases were only one or few eyelashes are affected lubrication (viscous eye drops), eye-lash trephination, laser ablation or electrolysis are often used. Removal of the affected eyelashes with a tweezer by an ophthalmologist may offer a cheap but temporary relief. Also, cryotherapy where the eye-lash follicle is destroyed using a cryogenic solution is another permanent but is a less straightforward and more costly procedure \(^\text{[123]}\).

Various surgical interventions are used for treating for more severe cases of trichiasis to reduce the risk of blindness. The procedure normally involves making an incision in the entropic lid, followed by making an outward rotation and suturing of the distal fragment in the corrected position \(^\text{[124]}\). However, the trichiasis often returns, with the recurrence rates ranging from 10% at 1 year to 60% at 3 years \(^\text{[125]}\). Factors which contribute to recurrence include preoperative disease severity, inherent limitations of the surgical procedure, the skills of the surgeon, wound healing responses, infection, and chronic inflammatory-fibrogenic responses \(^\text{[124]}\).

When significant scarring to the cornea has occurred penetrating keratoplasty can be conducted to save the vision. It is a full-thickness transplant procedure, in which full-thickness resection of the patient's cornea is carried out. The cornea is then replaced with a donor corneal graft. Interrupted and running sutures are then placed in radial fashion at equal tension to minimise post-operative astigmatis. The transplanted cornea can last for decades provided the trichiasis does not reoccur and cause scarring the new tissue as well \(^\text{[126]}\).

All these approaches are not always successful and are ineffective long term, in many cases. Such treatments require skilled specialists who are less accessible in endemic areas and are also costly \(^\text{[127]}\). So, there is a real clinical
need for treatments that are more accessible to patients to improve the outcome of the surgical treatment.

The issue is that surgery may add to the scarring process which develops during wound healing where the normal tissue architecture is disrupted. The normal architecture is replaced by excessive connective tissue through the abnormal accumulation of ECM components. Neutrophils play a role in trachoma pathophysiology by producing toxic reactive oxygen and nitrogen species that damage host tissue and can also produce matrix metalloproteinases (MMPs). Macrophages are also present in trachomatous tissue and may be involved in tissue damage in chlamydial infection [128].

MMPs are a group of enzymes which are normally required for normal tissue homeostasis, there is also evidence that they play a role in the pathogenesis of a range of inflammatory-fibrotic diseases. They disrupt the basement membrane and aid in the immune cell recruitment and have a wide range of effects on inflammatory and immune processes. These include modulating chemokine activity and activation of transforming growth factor β (TGFβ), interleukin-1β (IL-1β), and tumour necrosis factor α (TNFα) [128].

Scarring in trachoma shows a differential expression of MMP-7, -9, -10, and -12 and tissue inhibitor of MMP (TIMP)-1. The MMP1/TIMP1 transcript ratio alteration is associated with recurrence of trichiasis after surgical treatment [124]. The production of scar tissue in trachoma originates from activated conjunctival fibroblasts that are stimulated to produce collagen via several pro-fibrogenic mediators. These mediators include TGF-β, platelet-derived growth factor (PDGF), connective tissue growth factor (CTGF), and basic fibroblast growth factor (bFGF). Chemokines have also been shown to act as fibrogenic mediators and have been associated with scarring [128], [129].

The MMP-9 enzyme plays an integral part in trachoma pathology as it is part of the neutrophil lysosome and mediates the epithelial dissolution associated with the infection through degradation of type IV collagen. It is also a major effector of ECM turnover and is upregulated in the inflamed conjunctiva of trachoma subjects. It has been found that children with active trachoma have increased MMP-9 levels. Increased expression of MMP-9 and a coding single nucleotide polymorphism that is adjacent to the active binding site of the MMP-9 enzyme is also associated with adults with scarring trachoma [124].
Natividad et al reported that the dysregulation of the ECM proteolysis during tissue repair following infection and inflammation may play a major role in developing the fibrotic sequelae of infection. They found a genetic variation within the MMP-9 gene affects *in-vitro* MMP-9 expression levels, enzymatic activity and susceptibility to various inflammatory and fibrotic conditions. It is a Q279R mutation, found in exon 6 of MMP-9, which results in a nonsynonymous amino-acid change inside the active site of the enzyme. This reduces the MMP-9-induced ECM turnover during inflammatory episodes in trachoma and its associated fibrosis. Hence, it results in a lower risk of trachomatous scarring and trichiasis [130].

1.5.1.2 Wound Healing

Wound healing is a physiological process which forms a structural and functional barrier following injury. It functions to stop bleeding, prevent drying and reduce the chances of infection. The stages of wound healing (Figure 10) is divided into three overlapping phases: 1) haemostasis and inflammation; 2) proliferation; and 3) wound contraction and tissue remodelling. The wound healing stages are heavily disrupted in trachoma (trichiasis) [131].
Haemostasis involves the release of blood cells and plasma proteins from the damaged blood vessels, as well as exposure of the various ECM components of these connective tissues. This then leads to the initiation of the clotting cascade which act to stop the bleeding. Also, additional inflammatory cells and fibroblasts are attracted to the wound which in turn amplify inflammation at the site of injury [131], [132].

During the inflammatory phase, re-epithelialisation and granulation tissue formation occurs. The granulation tissue consists of fibroblasts, endothelial cells and macrophages. The fibroblasts deposit and remodel the wound ECM [131]. The final phase of wound maturation is the process of tissue remodelling, contracture and the formation of collagenous scar. This process involves ECM synthesis, deposition and remodelling. Fibroblasts are primarily involved in depositing and degrading collagen and arranging collagen fibres [131].
1.5.1.2.1 The Function of MMPs in the Body

MMPs are a group of enzymes that are essential for the degradation and remodelling of the ECM and are found in many tissues. The criterion for membership of this enzyme family is sequence homology with the catalytic domain of the first discovered MMP, fibroblast collagenase (MMP-1). All the MMPs share a catalytic domain that contains the zinc-binding motif, with the three histidines ligating the active Zn$^{2+}$ ion. MMPs are Zn$^{2+}$-dependent proteins and there is a considerable overlap in substrate specificities, and they appear to be involved in degradation of abundant ECM components.

Based on sequence homology and substrate specificities the MPP family can be divided into different groups. These MMP families can be broadly classified into collagenases, gelatinases, stromelysins, and the membrane-type metalloproteinases (Table 5). The structure of MMP-2 and -9 (Figure 12) is composed of where the catalytic site which contains three essential Zn$^{2+}$ binding sites. The haemopexin domain in the structure mediates interaction with enzyme substrates. The fibronectin-like domain, specific to gelatinases, facilitates substrate binding.

---

**Figure 11.** Schematic structure of the MMP-2 and MMP-9 enzyme. Adapted from Tveita, Rekvig, and Zykova 2008.
Table 5. The major members of the matrix metalloprotease family. Adapted from Hoekstra 2004 \[^{135}\].

<table>
<thead>
<tr>
<th>Group</th>
<th>Descriptive name</th>
<th>n</th>
<th>Principal substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagenases</td>
<td>Interstitial collagenase</td>
<td>MMP-1</td>
<td>Fibrillar collagen type I, II, II</td>
</tr>
<tr>
<td></td>
<td>Neutrophil collagenase</td>
<td>MMP-8</td>
<td>Fibrillar collagen type I, II, II</td>
</tr>
<tr>
<td></td>
<td>Collagenase-3</td>
<td>MMP-13</td>
<td>Fibrillar collagen type I, II, II</td>
</tr>
<tr>
<td></td>
<td>Collagenase-4</td>
<td>MMP-18</td>
<td>Fibrillar collagen type I, II, II</td>
</tr>
<tr>
<td>Stromelysins</td>
<td>Stromelysin-1</td>
<td>MMP-3</td>
<td>Proteoglycans, laminin, fibronectin, nonfibrillar collagen</td>
</tr>
<tr>
<td></td>
<td>Stromelysin-2</td>
<td>MMP-10</td>
<td>Proteoglycans, laminin, fibronectin, nonfibrillar collagen</td>
</tr>
<tr>
<td></td>
<td>Matrilysin</td>
<td>MMP-7</td>
<td>Proteoglycans, laminin, fibronectin, nonfibrillar collagen</td>
</tr>
<tr>
<td>Gelatinases</td>
<td>Gelatinase A (72kDa)</td>
<td>MMP-2</td>
<td>Gelatins, nonfibrillar collagen types IV, V</td>
</tr>
<tr>
<td></td>
<td>Gelatinase B (92kDa)</td>
<td>MMP-9</td>
<td>Gelatins, nonfibrillar collagen types IV, V</td>
</tr>
<tr>
<td>Membrane type</td>
<td>MT1-MMP</td>
<td>MMP-14</td>
<td>Progelatinase A, procollegenase-3</td>
</tr>
<tr>
<td></td>
<td>MT2-MMP</td>
<td>MMP-15</td>
<td>Progelatinase A</td>
</tr>
<tr>
<td></td>
<td>MT3-MMP</td>
<td>MMP-16</td>
<td>Progelatinase A</td>
</tr>
<tr>
<td></td>
<td>MT4-MMP</td>
<td>MMP-17</td>
<td>Progelatinase A</td>
</tr>
<tr>
<td></td>
<td>MT5-MMP</td>
<td>MMP-21</td>
<td>Progelatinase A</td>
</tr>
</tbody>
</table>

MMPs also act on cytokines, chemokines, receptors, cell-cell adhesion molecules, latent growth factors and anti-microbial peptides. They can either potentiate or inhibit their targets causing different biological effects depending on those targets. MMPs play a major role in cell growth, signalling, migration, differentiation and apoptosis. Additionally, MMPs are involved in other complex biological processes such as ovulation, remodelling of connective tissues and wound healing \[^{133}\]. Examples of the different biological effects of specific MMP enzymes are summarised in Table 6.
### Table 6. Biological effects mediated by the MMP cleavage of different substrates. Adapted from Nagase, Visse, and Murphy 2006 [136]

<table>
<thead>
<tr>
<th>Biological Effect</th>
<th>Responsible MMP</th>
<th>Substrate Cleaved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Keratinocyte migration and reepithelialisation</td>
<td>MMP-1</td>
<td>Type I collagen</td>
</tr>
<tr>
<td>Neurite outgrowth</td>
<td>MMP-2</td>
<td>Chondroitin sulphate proteoglycan</td>
</tr>
<tr>
<td>Osteoclast activation</td>
<td>MMP-13</td>
<td>Type I collagen</td>
</tr>
<tr>
<td>Adipocyte differentiation,</td>
<td>MMP-7</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>Cell migration</td>
<td>MMP-1, -2 and -3</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>Pro-inflammatory</td>
<td>MMP-1, -3 and -9</td>
<td>Processing IL-1β from the precursor</td>
</tr>
<tr>
<td>Anti-inflammatory</td>
<td>MMP-1, -2 and -9</td>
<td>IL-1β degradation</td>
</tr>
<tr>
<td>Anti-inflammatory</td>
<td>MMP-2, -3, -13</td>
<td>Monocyte chemoattractant protein-3</td>
</tr>
<tr>
<td>Tumour cell resistance</td>
<td>MMP-9</td>
<td>Intercellular Adhesion Molecule 1 (ICAM-1)</td>
</tr>
<tr>
<td>Thymic neovascularization</td>
<td>MMP-9</td>
<td>Collagen IV</td>
</tr>
</tbody>
</table>

#### 1.5.1.3 Potential Treatments

Azithromycin is a macrolide antibiotic characterized by a broad antibacterial spectrum, a long half-life related to its tissue and cell penetration, and anti-inflammatory properties [137]. Therefore, Burton et al used a single dose azithromycin in a randomised clinical trial after trichiasis surgery to prevent recurrence but it did not show any difference to the placebo [125].

Doxycycline is a tetracycline antibiotic which is commonly used to prophylactically and treat to bacterial and parasite infections, including *Chlamydia trachomatis*. Doxycycline has a similar efficacy to azithromycin in terms of its anti-inflammatory profile. More importantly doxycycline also acts an MMP inhibitor particularly MMP-9, at sub antimicrobial doses in patients. H. Li et al showed that doxycycline significantly reduced collagen matrix remodelling and contraction in a trichiasis-derived conjunctival fibroblasts
contraction assay. It inhibited mRNA expression of MMP-1, MMP-7, MMP-9, and MMP-12 during contraction. Doxycycline might be a good adjuvant treatment following trichiasis surgery. Its high half maximal inhibitory concentration (IC$_{50}$) value might present an issue in terms of potential dosing in humans, especially for ocular formulations.$^{[138]}$

Due to the underlying immune response and scarring process prior to the surgery, a more severe inflammation can therefore occur following surgery and may produce additional scarring and recurrent trichiasis. As a result, pharmacological inhibition of MMP enzymes may control the inflammatory scarring process before or after surgery.$^{[125]}$ MMP inhibitors can therefore help significantly improve the outcome of trichiasis as they are a critical target in the disease. As described earlier, the inhibition of MMPs would also inhibit the activity of conjunctival fibroblasts which also play a major role in the scarring process. Oral MMP inhibitors can have major side effects due to their systemic effects such as muscle and joint pain.$^{[139]}$ Some of the effects of inhibiting certain MMP enzymes described in Table 7 (next page). The table shows the effect of ablation of individual MMP genes in mouse models is shown. It shows that essentially inhibition/ lack of activity of certain MMPs can have serious consequences in animals. Examples of biological effects range from increased immune complex mediated arthritis to defects in skeletal development.

Krzeski et al reported that 35% of patients in a randomised clinical trial developed musculoskeletal adverse events when they received PG-116800 (a broad spectrum MMP inhibitor) orally for 1 year. There was no improvement in the symptoms of osteoarthritis in patient that PG-116800 was aiming treat.$^{[140]}$

Recently, eye drops with an MMP inhibitor which would act locally on the cornea were formulated and showed promising data in-vitro. The results show that formulation significant. It showed a significant reduction in the amount of gel contraction in-vitro when human tenon fibroblasts were incubated in a 3D collagen hydrogel.$^{[141]}$ Yye drops get eliminated from the rapidly from the target tissue due to mechanisms described earlier. Therefore, the frequency of topical application might be an issue that can affect compliance and adherence to the treatment and hence outcome.
Table 7. The major effects of ablation of MMP genes in mouse models of cancer, vascular disease and arthritis. Adapted from Murphy & Nagase, 2008 [136].

<table>
<thead>
<tr>
<th>Gene</th>
<th>In Vivo Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2</td>
<td>Reduction pancreatic tumorigenesis</td>
</tr>
<tr>
<td></td>
<td>Reduction of neointima formation on vascular injury</td>
</tr>
<tr>
<td></td>
<td>Protection from cardiac rupture post-myocardial infarction</td>
</tr>
<tr>
<td></td>
<td>Increased immune complex-mediated arthritis</td>
</tr>
<tr>
<td>MMP-7</td>
<td>Reduction in intestinal adenoma formation</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Reduction in pancreatic tumorigenesis</td>
</tr>
<tr>
<td></td>
<td>Reduction of neointima formation on vascular injury</td>
</tr>
<tr>
<td></td>
<td>Protection from cardiac rupture post-myocardial infarction</td>
</tr>
<tr>
<td></td>
<td>Decreased immune complex-mediated arthritis</td>
</tr>
<tr>
<td>MMP-11</td>
<td>Delayed mammary tumorigenesis</td>
</tr>
<tr>
<td></td>
<td>Accelerated neointima formation on vascular injury</td>
</tr>
<tr>
<td>MMP-12</td>
<td>Reduced macrophage numbers in ligament injury</td>
</tr>
<tr>
<td>MMP-13</td>
<td>Spontaneous abnormal growth plate, increased trabecular bone</td>
</tr>
<tr>
<td>MMP-14</td>
<td>Acceptation of mammary tumorigenesis, reduced metastases</td>
</tr>
<tr>
<td></td>
<td>Skeletal development defects, arthritis and connective tissue disease</td>
</tr>
</tbody>
</table>

1.5.1.3.1 Tissue Specific Anti-Scarring Depot

Potentially, a tissue specific treatment using MMP inhibitors directly after trachoma surgery may offer a much-needed improvement to the currently poor outcome for this condition. Therefore, an IPC could be an excellent candidate to prepare an in-situ gelling slow release depot of an anti-scarring agent which can be injected directly after trachoma surgery. The depot would be injected using a small gauge needle into the subconjunctiva (inside the eyelid) where the drug would slowly be released locally over time to prevent scarring in the eyelid. This limits systemic drug exposure and maximises the amount drug present at the site of action. As the depot would be administered at the time of
surgery there would be no further action required by the patient. The collagen in the IPC may potentially provide an extra support to the eyelid while it degrades very slowly over time.

Hydrogels are thought to be an excellent candidate vehicle for the tissue specific delivery of anti-scarring agent for trachoma. Hydrogels present some limitations including difficulty in handling (mechanically weak) and challenges for loading of therapeutics \cite{93}. Using an in-situ gelling hydrogel like an IPC can potentially overcome these issues. Anti-scarring molecules can be mixed with the IPC as a solution or as a suspension of drug particles. The formulation would undergo a solution–gel transition inside the human body, resulting in a depot which takes the shape of the available space at the injection site causing minimal discomfort \cite{90}. The preparation can be injected directly after the surgery or even be used to reduce the need for surgery.

The EDTA present in the IPC may have an agonistic or additive effect to the formulation. EDTA is a broad spectrum MMP inhibitor can permanently inhibits the activity of the enzymes. MMPs require calcium ions to maintain their tertiary structure and zinc ions to maintain functional active sites. Both ions can be chelated by EDTA which results in the enzyme inactivation \cite{142}. As a result, the EDTA in an IPC might be able to aid in the treatment of trichiasis by inhibiting MMPs such as MMP-9 and therefore reduce the scarring process by for instance slowing down the ECM turnover as well as inhibiting the degradation of collagen in the IPC. The tissue MMPs will also act degrade the collagen in IPC and therefore potentially lessening their effects on the ocular tissues.

The IPC hydrogel would be ideal for delivering micro- or nanoparticles containing anti-scarring drugs into the subconjunctiva. The conjunctiva (Figure 12) is a thin membrane covering the sclera (bulbar conjunctiva) and the inside of the eyelids (palpebral conjunctiva) \cite{143}.
By entrapping the microparticles inside the gel, the IPC would retain the microparticles at the injection site. Additionally, by coating/entrapping the particles inside an IPC, there would potentially be protection from a localised foreign body response which would drive scarring. There may be a need to minimise protein encapsulation phagocytosis by macrophages of particulate drugs. The depot system can potentially maintain optimal therapeutic drug levels and reduce drug related adverse events by localising the drug exposure. Finally, the depot could improve treatment outcome further by maximising and prolonging drug exposure at the target site. A depot introduced at the time of surgery would also avoid frequent dosing using eye drop formulations [116]. The subconjunctival administration would be into the superior palpebral conjunctiva (Figure 13) as trichiasis primarily affects the upper eyelids.


1.6 Hypothesis and Aims

Much research is dedicated to developing biomedical materials that can be utilised by minimally invasive techniques. Collagen is a good candidate for further development. It is hypothesised that an injectable collagen hydrogel can be developed for use in drug delivery and pharmaceutical research.

The aims of this project include the characterisation of different sources of collagen type I to gain a better understanding of collagen physical, chemical and biological properties for use in biomedical applications. Atelocollagen reduces the immunogenicity risk, however, there is some evidence that suggests that the lack of telopeptides affects how collagen behaves in 3D culture environments. Therefore, atelocollagen will be studied further both in terms of its mechanical properties and its behaviour in 3D culture compared to telocollagen. As described earlier collagen type I properties and functions vary depending on the tissue it originates from. Thus, it is important to further analyse the differences between collagen extracted from different tissues to further improve the development of collagen-based biomaterials. Other biomaterials will be examined such as hyaluronic acid to determine changes in the mechanical properties of collagen gels and to prepare 3D cell cultures.

Another key aim is to explore a preparation method for producing an in-vivo biomimetic artificial vitreous substitute by crosslinking collagen type I and HA (HA or HA-tyramine). The possibility of in-situ crosslinking to prepare the vitreous substitute will also be explored to allow for ease of administration. Such substitutes may overcome the limitations of current artificial vitreous substitutes which include cataract and the requirement for a second surgery to remove the silicon oil. A HA-collagen hydrogel may also have utility as a simulated vitreous fluid for use in pharmaceutical development of intraocular medicines.

An in situ polymerised collagen (IPC) strategy will be optimised to develop a method for making IPC solutions with tuneable mechanical properties. The aim is to formulate the IPC for drug and tissue specific delivery. An IPC will first be examined to formulate an injectable in-situ gelling implant depot designed for the treatment of trichiasis after surgery. The aim is to formulate a slow release anti-scarring agent such as an MMP inhibitor (e.g.}
ilomastat) which can be injected using a small gauge needle designed to mediate post-surgical healing.

A formulation method will be established using different parameters to prepare a stable slow release depot with a tuneable release profile. Several formulation methods will be explored including the incorporation of the IPC with the anti-scarring drugs as solutions and as suspensions and using drug encapsulated microparticles. The formulations will be analysed physically, chemically and by *in-vitro* biological assays. The drug release will be measured using an *in-vitro* ocular model which mimics subconjunctival drug release.

The IPC will also be examined to formulate an injectable depot slow release system for macromolecules (e.g. bevacizumab) designed for subconjunctival and intraocular use. Different preparation methods will be investigated to prepare the IPC macromolecule formulation. The formulations will be evaluated for stability and antibody release using a biorelevant *in-vitro* ocular release model. Finally, the IPC will be studied further for use as a vehicle for the delivery of cell therapies for ocular diseases using human tenon fibroblasts (HTF) as a model cell line. A preparation method will also be developed, and the system will be analysed using different *in-vitro* techniques and bioassays.
Chapter 2: Preparation of Collagen-Based Biomaterials for Regenerative Medicines
2. Preparation of Collagen-Based Biomaterials for Regenerative Medicines

2.1 Introduction

Collagen type I is one of the most commonly used biomaterials used in regenerative medicines both clinically and experimentally. Different sources of collagen are used in many experimental studies without there being sufficient effort to determine the effect of the collagen source on mechanical properties. It has been shown that collagen in different parts of the body has different properties [37], [144]. Acid soluble (AS) bovine corium and rat-tail collagen type I are commonly used for preparing 3D constructs in both in-vitro and in-vivo studies. The rigidity and mechanical properties of the 3D environment of cells affects cell behaviour and signalling. Characterisation of collagen based hydrogel constructs from different collagen sources (corium or tendon) is therefore important to establish more in-vivo biomimetic environment for cells to grow [145].

The effect of lack of telopeptides on collagen type I (atelocollagen) on the mechanical properties and cell behaviour has not been well studied. There is some evidence that to suggest that lack of telopeptides affects collagen properties. In pepsin soluble (PS) collagen most of the telopeptides have been cleaved [24]. Cell alignment in tissues and the ECM is essential in human biological processes such as wound healing where collagen plays a major role in the process. In certain biomedical applications such as peripheral nerve repair, the need to guide nerve growth cell realignment is carried out prior to implantation [146]. Atelocollagen provides a smaller risk of immunogenicity and therefore its effect on cell realignment needs to be further analysed [29].

Hyaluronic acid (HA) is widely investigated in tissue engineering and drug delivery applications. HA substituted tyramine (HA-T) can be photolytically or enzymatically crosslinked using horseradish peroxidase (HRP) in the presence of hydrogen peroxide (H₂O₂) and reactive oxygen species (ROS). Covalent crosslinks are formed between the tyramine aromatic rings [67] and crosslink density is controlled by the extent of tyramine substitution on HA, which is often in the range of 1-5%. It may be possible that
HA-T can be mixed with collagen prior to crosslinking to further tune the mechanical properties of cell based hydrogels [68].

The ageing vitreous can cause loss of integrity resulting in retinal detachment. Vitreous substitutes (e.g. gases and silicon oil) can result in complications and a more biomimetic substitute is required [51]. Preclinical pharmaceutical studies for intraocular medicines designed for long duration of action also require a vitreous substitute that can provide a more biomimetic environment for studying intravitreal formulations.

HA is found in the vitreous humour (VH) at around 4:1 ratio with collagen type II [47]. A hydrogel based vitreous substitute composed of collagen and HA may mimic the physicochemical properties of the native VH. Carbodiimide mediated coupling reactions are often used to crosslink collagen and HA [147], [148]. Photochemical crosslinking has been used to crosslink collagen and HA-T [67].

Photochemical crosslinking of collagen type I in the cornea using riboflavin 5’ phosphate is routinely utilised for keratoconus with no cytotoxicity being observed. Keratoconus is caused by corneal thinning where the chemical crosslinking strengthens the cornea [64], [65]. For collagen the photolytic crosslinking has been described to occur between threonine, tyrosine, hydroxyproline and histidine amino acids. The reaction can also occur between the imidazole groups in these molecules (apart from threonine) [66]. Collagen can possible be crosslinked with HA through the phenol group of tyramine in HA-T and tyrosine in collagen. Photochemical crosslinking of collagen and HA-T is a potential method for preparing an artificial vitreous substitute which can be crosslinked in-situ allowing for ease of administration or in situ within a pharmaceutical preclinical 2 compartment model.

2.1.1 Aims

One of the aims and objectives of the study is to prepare different sources of collagen and telo- and atelocollagen hydrogels. This will be to analyse the effect of such differences on the mechanical properties and cell behaviour of such hydrogel constructs. An enzymatically (by HRP) crosslinked HA-T hydrogel will be prepared, characterised and embedded with cells to establish
whether such hydrogel could be mixed with collagen and to tune the mechanical properties of collagen hydrogels.

A method for preparing an artificial vitreous hydrogel substitute will be established and characterised. Both the carbodiimide coupling reaction and photochemical crosslinking will be used to prepare the hydrogel.

2.2 Materials and Methods

2.2.1 Materials

The following materials were purchased from commercial sources. Acid and pepsin soluble type I bovine collagen (Collagen Solutions, UK), rat-tail type I collagen (First Link, UK), hyaluronic acid (Lifecore, US), hyaluronic acid-tyramine (Lifecore, US), N-hydroxysulfo succinimide sodium salt (Alfa Aesar, US), Celltiti-Glo 3D (Promega, US), MMP Activity Assay Kit (ab112147, Abcam, UK), 24-well and 96 -well plate (Thermo Fisher Scientific, UK), Al(III) Phthalocyanine chloride disulfonic acid (adjacent isomer) (Frontier Scientific, US), F7 and ARPE-19 cells were kindly donated by UCL Institute of Ophthalmology (London, UK) and HDFa cells were kindly donated by UCL School of Pharmacy (London, UK). Foetal bovine serum, Penicillin-streptomycin, Dulbecco's Modified Eagle's medium (41965039), Minimum Essential Medium (10x, 21430020) were obtained from from GIBCO, UK. Sodium hydroxide; 4-morpholineethanesulfonic acid sodium salt; 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride, riboflavin 5’ phosphate sodium salt hydrate, phosphate buffered saline (PBS) tablets, female luer-luer coupler, dimethyl sulfoxide were all purchased from Sigma-Aldrich, UK.

2.2.2 Instrumentation

Bohlin Gemini HR Nano Rheometer (Malvern, UK); BioTek Synergy HT (BioTek Instruments, US); FEI/Thermo Quanta 200F scanning electron microscope (FEI, USA); Spectrum M2 (Molecular Devices, US) plate reader; Sorvall Legend RT+ Centrifuge (Thermo Scientific, US); Viva 6 centrifugal concentrator (30 kDa, Sartorium Stedium Biotech); Perkin Elmer Spectrum 100 FT-IR Spectrometer (Park Elmer, US); mounted LED (M470L4 Thorlabs
Ltd, UK); mounted LED (MM660L4, Thorlabs Ltd, UK); VirTis AdVantage freeze dryer (SP Scientific, US).

2.2.3 Methodology

2.2.3.1 Preparation of Collagen and HA-T Hydrogels

2.2.3.1.1 Collagen Hydrogel Preparation

Collagen solutions of bovine corium (acid and pepsin soluble) of rat-tail origin were used at 5 mg/mL to prepare a mixture of 80% collagen, 10% Minimum Essential Medium (MEM, 10×) and 10% of complete DMEM media. While working on an ice block, the collagen solution (800 µL, 5 mg/mL) was added to MEM (100 µL, 10×) in a bijou tube. The mixture was neutralised by adding 1 N NaOH which was noted by the change in MEM colour from yellow to peach (neutral pH). Complete DMEM media (10% FBS and 5 mM penicillin/streptomycin (P/S)) was added (100 µL) and the mixture was quickly mixed before pipetting the solution (500 µL) into 24 well-plates followed by incubation in a humidified incubator at 37°C and CO₂. The gelling time was then monitored. Gel formation was visually determined when the solution turned from clear to opaque (insoluble fibrils).

2.2.3.1.2 HA-T Hydrogel Preparation

HA-T gels were prepared according to the manufacturer’s instructions. Different concentrations (2.5 and 5 mg/mL) and levels of tyramine substitution (1.3 and 5%) of HA-T solutions were prepared. HA-T powder (1.25 mg or 2.5 mg) was dissolved in horseradish peroxidase (HRP) solution (500 µL, 10 U/mL in PBS, pH 7.4). The mixture was stored in the fridge (at 4°C) for 24 h to allow hydration of HA-T before addition of 20 µL (1:25, H₂O₂ solution) of 1.2 µM H₂O₂ to crosslink the solution to form the gel (500 µL). The mixture was quickly mixed with a pipette to distribute the H₂O₂ solution resulting HA crosslinking.

2.2.3.2 Mechanical Characterisation of Collagen and HA-T Hydrogels

Dynamic mechanical analysis (mechanical stiffness) was conducted on the prepared gels of HA-T and collagen (500 µL) with a rheometer using the
oscillometry function in rheometry. The strain was set to 5% (based on an amplitude sweep) and the frequency range was set to 0.1-10 Hz. Parallel plates (PP20) were used with a 400 µm gap and the temperature set at 37°C.

2.2.3.3 Cell Behaviour of Cells Grown in Collagen and HA-T Gels

2.2.3.3.1 General Cell Culture Protocol
The cell lines (e.g. HDFa, F7 and ARPE-19) were thawed from liquid nitrogen and were cultured in T75 culture flasks in complete Dulbecco's Modified Eagle's medium (DMEM) in 100 IU/ mL penicillin and 100 µg/mL streptomycin (P/S) and 10% foetal bovine serum (FBS) at 37°C with 5% CO₂ in a humidified incubator. The media was changed every 3 days. At approximately 80% cell confluency the cell lines were trypsinised and maintained routinely in the above media.

The trypsinisation process involved first removing the media from an 80% confluent T75 flask followed by adding trypsin EDTA (0.25%, 3 mL). The flask was then incubated for 4 min at 37°C and 5% CO₂. Then complete DMEM media (9 mL) was added to the flask and the solution was then subsequently transferred into a centrifuge tube. The tube was then centrifuged for 5 min at 1200 rpm and at an ambient temperature. The media was then discarded, and the cell pellet reconstituted in complete DMEM and cultured in a T75 flask. The flask was then incubated at 37°C and 5% CO₂ in a humidified incubator with regular media changes.

2.2.3.3.2 Collagen Gels

2.2.3.3.2.1 Contraction Assay
A contraction assay (Figure 13) was carried out using HDFa and F7 cells with the method was adapted from Davidenko et al [149]. Bovine corium acid soluble (AS) and pepsin soluble (PS) collagen and rat-tail collagen solutions were used at 5 mg/mL. While working on an ice block, MEM (10×) solution (100 µL) was added to a collagen solution (800 µL) and the mixture was neutralised using NaOH as described earlier (section 2.2.3.1.1). Cell suspension (100 µL, 40 million cells/mL) of HDFa (passage 7-11). The resulting mixture (75 µL, 300,000 cells/ well) was quickly pipetted into 96 well plates. To form the gels,
the plate was then incubated in a humidified incubator at 37°C and 5% CO₂. Complete DMEM (75 μL) was added into the wells. The same protocol was used to F7 cells (passage 16-20) embedded collagen gels and the different collagen sources. For the negative control, complete DMEM was supplemented with 10% bovine serum albumin (BSA) to inhibit gel contraction.

To detach the gels from the plate, a 10 μL pipette tip was moved around the well edges until the gel was seen to float. The plate was then photographed at time 0 and after 24 h, to measure the extent of gel contraction (collagen fibrils’ level of alignment by cells). The extent of gel contraction was monitored by digital photography (Samsung S8) where the areas were measured using the ImageJ software. The contraction was plotted as a percentage of gel diameter normalised to original diameter. This was done by dividing the diameter of the gel at each time point by the gel diameter at day 0 and then multiplying the answer by a 100.

![Figure 13](image.png)

**Figure 13.** A schematic description of the collagen contraction assay preparation process. After the cell embedded cell gel is set the media is added followed by gel floating. Adapted from Rosser 2011 [41].

### 2.2.3.3.2.2 Cell Viability

The cell viability of HDFa cells (passage 7-11) embedded in different collagen gels (PS, AS and rat-tail) was compared to those grown in a 2D environment (control). The gel embedded cells (3D cultures) were prepared as described earlier but the seeding cell density was 133,000 cells/mL (75 μL, 10,000 cells/well). This cell density is commonly used in cell viability assays [150]. The cells were incubated for 24 h without floating the gels at 37°C and 5% CO₂. The control was cells seeded (10,000 cells/well) into the wells (grown into 2D) at same conditions with the same amount of media cells. Controls of media
only and collagen gels only were prepared to account for background luminescence.

CellTiter-Glo® assay was utilised next to measure the cell viability after 24 h. CellTiter-Glo reagent (75 μL) was added to each well and the plate was shaken for 5 min at 250 rpm at an ambient temperature and then incubated for a further 25 min at the same temperature. The luminescence was then measured using a plate reader.

CellTiter-Glo® assay directly quantifies the amount of adenosine triphosphate (ATP) present within cells (Figure 14) The CellTiter-Glo assay is therefore an indicator of metabolically active cells; whereby a viable cell is more metabolically active and hence produces more ATP than does a less viable dying cell. The luminescence is a measure of the metabolic activity which is itself a marker of cell viability [151]. The Assay Guidance Manual describes that the measurement of ATP using firefly luciferase as a very commonly applied method for estimating the number of viable cells [152]. Moreover, the CellTiter-Glo® assay has been widely used in the literature as a measure of cell viability in various studies [153]-[157]. For example, Petty et al reported that ATP based assays that utilise firefly luciferase are excellent candidates for determining the number of viable cells [158]. In this thesis CellTiter-Glo assay was therefore be used to measure the % cell viability of cells.

**Figure 14.** CellTiter-Glo® assay chemistry and mechanism of action. The cell is the source of ATP in the luciferase reaction, which is responsible for the luminescence. Reproduced from Corporation 2015 [151].
2.2.3.3 HA-T Based 3D Cultures

Different concentrations (2.5 and 5 mg/mL) and levels of tyramine substitution (1-5%) of HA-T solutions were prepared. To HA-T powder (2.5 mg or 5.0 mg) was dissolved in horseradish peroxidase (HRP) solution (800 µL, 10 U/mL in PBS, pH 7.4). MEM (10x) (100 µL) was added to the solution. A cell suspension (100 µL 1.3 Million cells/mL) was mixed into the mixture. The solution (75 µL, 10,000 cells/well) was pipetted into 96 well plates. H$_2$O$_2$ solution (3 µL, 1:25) was pipetted into the wells and quickly mixed using a 10 µL pipette tip. The plate was incubated for 15 min at 37°C. Complete DMEM (75 µL) was added to each well and the plate was incubated for 24 h in a humidified incubator for 24 h at 37°C and 5% CO$_2$. The cell viability was measured using CellTiter-Glo® assay as described earlier.

A control of 3D cultures of rat-tail collagen gels (commonly used in 3D cultures) were prepared as described in the cell viability study earlier. Using the conditions, HA-T gels were prepared without any cells (a negative control) to account for any background luminescence.

2.2.3.3.1 H$_2$O$_2$ Dose-Response Curve

A cells suspension of HDFa (passage 7-11) and F7 (passage 16-20) cells (75.0 µL, 10,000 cells/well) were pipetted into 96 well plate. Solutions of H$_2$O$_2$ (3.0 µL, 1:25) were added to wells at different H$_2$O$_2$ concentrations (0.001-3.75 µM). The solutions were mixed using a pipette tip. A control of cells only wells without exposure to H$_2$O$_2$ were prepared. Wells of complete DMEM and 3.75 µM were prepared as a control to account for background luminescence. The plate was incubated for 24 h humidified incubator at 37°C and 5% CO$_2$. The cell viability analysed using a CellTiter-Glo® assay as described earlier (section 2.2.3.3.2.2).

2.2.3.4 Artificial Vitreous Preparation and Characterisation

2.2.3.4.1 Carbodiimide Coupling Reaction

Although not always efficient, the carbodiimide coupling reaction can be conducted in aqueous buffer solutions. Two methods were evaluated. Collagen and high molecular weight HA (~1.8 MDa) were mixed at different
ratios of collagen: HA for both reaction methods (catalysed by carbodiimide and NHS). As this was a proof of concept study, a triplicate of samples was prepared one time.

**Method 1:** Bovine collagen type I solution was concentrated from 6.0 mg/mL (15.0 mL) to 10.0 mg/mL (9.0 mL) using a centrifugal concentrator (30.0 kDa molecular weight cut off). The concentrator was centrifuged at 4000 rpm, at 4°C. The collagen solution (62.5 µL) was then mixed with 2.5 mg/mL HA (937.5 µL, pH 4), at a 1:4 ratio (collagen: HA). In the resulting solution (1.0 mL), sodium chloride (NaCl, 9.0 mg) and 2-(N-Morpholino) ethanesulfonic acid (MES) sodium salt (20.0 mg) were dissolved. Next, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC, 9.8 mg) and N-hydroxysulfosuccinimide sodium salt (NHS, 11.1 mg) were then dissolved in the solution. A solution of 1 N NaOH (20.0 μL) was added and the solution was mixed by syringe-to-syringe passing using a luer-luer connector (syringe mixing method). This method (**Figure 15**) is used to mix solution with high viscosity. The reaction solution was transferred to a centrifuge vial and then centrifuged for 5 min at 4000 rpm to remove air bubbles. The reaction was then left to proceed for 18 h at 4°C.

![Syringe Mixing Method](image)

**Figure 15.** A visual representation of the syringe mixing method using two syringes and a female luer-luer connector.

**Method 2:** Collagen solution (62.5 µL, 10 mg/mL) and HA (937.5 µL, 10 mg/mL) were mixed and the mixture subsequently lyophilised for 72 h using a freeze drier. The reaction was carried out as described by Davidenko et al [148]. The freeze-dried cake was immersed in 95.0% ethanol solution (in 5.0% DI
water) containing 33.0 mM of EDC and 6.0 mM of NHS for 4 h at 25 °C. After crosslinking, the scaffolds were washed thoroughly in DI water (5x, 5 min).

2.2.3.4.1.1 Chemical and Physical Characterisation

Photographic images were taken before and after the reaction to visualise the difference in gel stiffness. Each sample was air-dried and then analysed by Fourier-transform infrared spectroscopy (FTIR) using an attenuated total reflectance sample holder. A few milligrams of the desired sample were placed in a sample holder and an even force of 130 gauge was applied onto the sample. The IR spectrum was collected over the range of 650-4000 cm\(^{-1}\) at a resolution of 1 cm\(^{-1}\) using 20 scans. The spectra were compared with the standard characteristic IR absorption frequencies of organic functional groups to identify the chemical composition of each sample \(^{[159]}\).

To characterise the gel viscosity, a plate-plate rheometer was used and parallel plates (PP20) were used, with the gap set to 400 µm and the sheer rate was set to a range of 0.1-100 s\(^{-1}\), at 37°C.

2.2.3.4.2 Photochemical Crosslinking

2.2.3.4.2.1 Preparation

HA-T photochemical crosslinking was carried out using riboflavin 5’ phosphate sodium salt hydrate (Figure 16) at 25 and 50 µg/mL, adapted from Donnelly et al \(^{[67]}\). Solutions of HA-T (1-5 mg/mL in PBS 7.4), with different tyramine substitutions (1, 3 and 5%) were prepared in different riboflavin concentrations (25 and 50 µg/mL). Also, solutions of PS bovine corium type I collagen (1-5 mg/mL in PBS 2x 7.4) prepared. A solution mixture of collagen:HA-T was prepared at a 1:4 ratio (0.675 mg/mL collagen:2.5 mg/mL HA-T). For example, riboflavin phosphate (2.5 mg) was dissolved in PBS (1 mL). This solution (10 µL) was diluted in PBS (990 µL, 1:100) to prepare 25 µg/mL riboflavin. In a vial was dissolved 1% HA-T (2.5 mg) into the diluted riboflavin solution (1 mL) and left fully to hydrate overnight at 4°C. The solution (500 µL) was pipetted into a 24 well plate. The plate was then illuminated at 4 and 8 mW/cm\(^2\) using a mounted LED (965 mW power output) at 470 nm. To achieve 4 mW/cm\(^2\), the
lamp was set at 16 cm from the plate and 4 cm to achieve 8 mW/cm$^2$. The crosslinking time was varied from 10 min to 1 h at ambient temperature.

For controls, solutions of HA-T, collagen and riboflavin only were illuminated. The effect of gel thickness on the crosslinking was also tested by varying the solution volume from 250 µL to 1 mL in a 24-well plate. To test the possibility of crosslinking the macromolecules in the red light spectrum, different concentrations (25 µg/mL to 1 mg/mL in PBS, pH 7.4) of Al(III) Phthalocyanine chloride disulfonic acid (adjacent isomer) (AlPcS2a) were used instead of riboflavin 5’ phosphate (Figure 16) to crosslink the macromolecules. The HA-T and collagen solutions where prepared and crosslinked as described above. The reaction was conducted at 660 nm using a mounted LED (1050 mW power output) at 4 and 8 mW/cm$^2$ for 10 min-1 h and at ambient temperature. The same lamp to plate distance used in the riboflavin experiments were utilised.

![Chemical Structures](image)

**Figure 16.** The chemical structure of two ROS generators: A), Al(III) Phthalocyanine chloride disulfonic acid (adjacent isomer) and B) Riboflavin 5’ phosphate sodium salt hydrate.
2.2.3.4.2.2 Mechanical Properties

To characterise the viscosity of the photochemically crosslinked gels (500 µL) a plate-plate rheometer utilised as described in section 2.2.3.4.1.1.

2.2.3.4.3 In-vitro Biocompatibility

The effect of in-situ crosslinking on the viability adult retinal pigment epithelial cell line-19 (ARPE-19) was tested. A cell suspension (75 µL, 10,000 cells/ well) of ARPE-19 cells (passage 29-32) were plated into wells in a 96 well plate. The plate was incubated in a humidified incubator for 16 h at 37°C and 5% CO₂. The media was replaced with a solution (75 µL) of HA-T and collagen in riboflavin (25 µg/mL). HA-T solutions (2.5 and 5 mg/mL in 25 µg/mL riboflavin) were prepared in 90% PBS and 10% MEM (10x), at all tyramine substitution levels. A solution of HA-T-collagen (1:4, 0.675 mg/mL: 2.5 mg/mL) in 90% PBS and 10 % MEM (10x) in riboflavin (25 µg/mL) was prepared. The plate was illuminated at 4 mW/cm² as described earlier (Figure 17). The gel was quickly washed with complete DMEM (3×) and complete DMEM (75 µL) was added the plate was incubated for 24 h at 37°C and 5% CO₂ in a humidified incubator.

For controls, cells in complete DMEM were illuminated with blue light for 15- and 30 min. Solutions of HA-T (5 mg/mL and 25 µg/mL in complete DMEM) at all substitution levels and the HA-T: Col (4:1) solutions were incubated with the plated cells without light illumination. The controls were incubated under the same conditions and for the same time length as the rest of the samples. The cell viability was measured using CellTiter-Glo® assay as described earlier in section 2.2.3.3.1.2.
Figure 17. A schematic description of the biocompatibility study setup. The addition of the biomaterials was followed by the illumination stage.

2.2.3.5 Statistical Analysis

All experiments in this thesis were conducted in triplicate samples and on at least three separate occasions (henceforth denoted by ‘n=3’), unless otherwise stated. The mean of each value was then calculated from the sample data. Depending upon the specific data analysed, statistical significance was tested using a One-way Repeated Measures ANOVA (ORM-ANOVA) or Factorial ANOVA (F-ANOVA). ORM-ANOVA is also known as within subject ANOVA and is used to compare the means of three or more matched groups. In this chapter, ORM-ANOVA was used to analyse the cell viability data (apart from the hydrogen peroxide dose response curve data). F-ANOVA compares the mean differences between two or more groups (the independent variables) (e.g., different drugs) that have a within-subject factor that has been measured on more than one occasion (e.g., time or different sites). F-ANOVA was the most suitable statistical test to analyse the data in this chapter because the dependent variable was continuously measured and had been measured on X occasions. The data tested for statistical significance was analysed using IBM SPSS version 25.

Error bars represent the standard error of the mean (SE), which is a statistical test that denotes how far the sample mean deviates from the actual population mean. The SE uses the standard deviation (SD), the amount of variability from the mean, to measure the accuracy within which a sample distribution represents a population [160]. Both the SE and SD can be used to represent error bars. However, SE also has the additional benefit of accounting for the number of experimental repeats and thus how far the sample mean
deviates from the actual population mean \cite{161}. The SE was utilised in this thesis as the n number was at least equal to 3 where each n number contained at least three replicates (i.e. n=3). A minimum significance level of p< 0.05 was used for all statistical tests \cite{160}.

2.3 Results

2.3.1 Collagen Gels Mechanical Properties and Morphology

To compare the mechanical strength of the hydrogels, the mechanical stiffness was measured using a plate-plate rheometer \cite{37}. DMA measures the dynamic modulus (also known as the complex modulus/G') which describes the mechanical stiffness of a material that is resistant to deformation when subjected to a given stress. Stiff materials are needed to transmit forces (e.g. in tendons and ligaments) and to resist deformation \cite{37}, \cite{162}. To obtain the complex modulus, a compressive stress is applied to the material at a specified temperature and different loading frequencies. The applied stress and the resulting axial strain of the specimen are measured and used to calculate the dynamic modulus. The complex modulus is defined as the peak stress divided by the peak strain at a specific frequency and temperature combination \cite{162}. The complex modulus (G'), is also defined as the ratio of the amplitude of the sinusoidal stress at any given time (t) and the angular load frequency, ω, δ = δ₀\sin(ωt) and the amplitude of the sinusoidal strain ε = ε₀\sin(ωt-φ), at the same time and frequency, that results in a steady state response. It can be described by the equation below \cite{163}:

\[ G' = \frac{\delta}{\varepsilon} = \frac{\delta_0 e^{i\omega t}}{\varepsilon_0 e^{i(\omega t-\phi)}} = \frac{\delta_0 \sin(\omega t)}{\varepsilon_0 \sin(\omega t-\phi)} \]

- δ₀ = peak (maximum) stress
- ε₀ = peak (maximum) strain
- φ = phase angle, degrees
- ω = angular velocity
- t = time, seconds
- i = imaginary component of G'
The gelling time and the mechanical stiffness (complex modulus) of collagen gels was analysed to study the effect of the collagen source and telopeptides on the collagen gel properties. The collagen source and telopeptides had a significant effect (p < 0.001) on gelling time. Tendon derived collagen hydrogel was mechanical stronger than corium derived collagen hydrogels. The telocollagen (AS collagen) hydrogel was mechanical stiffer than an atelocollagen hydrogel. Gelling times for AS rat tail, AS and PS bovine corium to form a hydrogel were 10, 15 and 25 min respectively. The collagen preparation had a significant effect (p< 0.001) on the mechanical stiffness of gels. The mechanical stiffness (Figure 18) of all three collagen gel preparations increased (more than doubled) with the rise in oscillation frequency. The oscillation frequency had significant effect (p< 0.001, within subject F-ANOVA) on the mechanical stiffness. The PS bovine collagen was visibly weaker and did not maintain its shape. A gel of AS rat tail collagen was 10× stiffer than PS bovine corium collagen and 1.5× stiffer than AS bovine corium. AS bovine corium collagen was 6.4× stiffer than PS bovine corium collagen gel. For example, at 1.3 Hz the mechanical stiffness was 12.0, 68.0 and 110.7 Pa for PS bovine corium, AS bovine corium AS rat-tail collagen gels respectively.

Figure 18. The modulation in the gel mechanical stiffness (complex modulus) of collagen gel preparations of bovine and rat-tail origin at 37°C. AS= acid soluble collagen and AS= pepsin soluble collagen, at 5 mg/mL collagen. The values represent the mean ±SE at n=3. p < 0.001 when the three different preparation profiles were compared using F-ANOVA. *= Statistically significant (p < 0.05).
2.3.2 HA-T Gels Mechanical Properties

HA-T gels were prepared to compare their mechanical properties to collagen gels and to investigate whether they can be used to grow cells. The gels were enzymatically crosslinked (Figure 19) using HRP enzyme H₂O₂ which utilise the phenol groups of tyramine for the crosslinking process [68]. The effect of tyramine substitution level on HA-T gels (5 mg/mL) was evaluated (Figure 20). The mechanical stiffness increased with the rise in oscillation frequency (p< 0.001). The increase in mechanical stiffness was due to the diffusion of the water molecules out of the gel structure due to the increased vibration frequency which in turn resulted in the HA-T molecules being closer together and hence able resist deformation. Tyramine substitution had a significant effect (p= 0.026) on the mechanical stiffness (complex modulus). At 1.3 Hz, the gel mechanical stiffness was 131.0, 131.0 and 147.2 Pa for 1, 3 and 5 % tyramine respectively.

Figure 19. The reaction mechanism of the HA-T crosslinking by H₂O₂, oxidised by the HRP enzyme. Reproduced from Darr and Calabro 2009 [4].
Figure 20. The effect of tyramine substitution levels on the mechanical stiffness (complex modulus) of 5 mg/mL HA-T hydrogels at 37°C. Tyramine substitution levels were 1-5%. The values represent the mean ±SE, at n=3. All p values >0.05 when the different substitution levels were compared using F-ANOVA. N/S = Not statistically significant (p > 0.05).

The mechanical stiffness was concentration-dependent (Figure 21) for all the HA-T gels with different tyramine substitutions. For example, at 10 Hz the mechanical stiffness was around 500 Pa for the 10 mg/mL gels but around 290 Pa for the 5 mg/mL gels. The mechanical stiffness almost doubled with the increased polymer concentration (p < 0.001) regardless of the tyramine substitution level. The complex modulus increased with the change in oscillation frequency for all HA-T preparations (p < 0.001).
Figure 21. The effect of increasing the HA-T concentration on the mechanical stiffness (complex modulus) of HA-T hydrogels. Preparations were at 5 and 10 mg/mL at 1-5% tyramine substitutions and analysed at 37°C. The values represent the mean ±SE at n=3. p values < 0.001 when comparing the two concentration profiles of the HA-T gels using F-ANOVA. * = Statistically significant (p < 0.05).

When the mechanical properties of HA-T gels are compared to collagen (Figure 22) it was observed that HA-T gels were stiffer than both AS and PS bovine corium collagen (p< 0.001), at 5 mg/mL. The mechanical stiffness of HA-T gels was also significantly different to the AS rat-tail collagen gel (p< 0.001) even the complex modulus values were closer to the HA-T gels than the bovine corium collagen gels. It was observed that the complex modulus of HA-T gels increased in a similar profile to the collagen gels but at a much lower rate.
Figure 22. The difference in the mechanical stiffness (complex modulus) of HA-T and collagen hydrogel at 37°C. The hydrogels’ concentration was 5 mg/mL and HA-T preparations were at 1-5% tyramine. AS=acid soluble and PS=pepsin soluble collagen. The values represent the mean ±SE at n=3. p values< 0.001 when the HA-T hydrogels were compared to the collagen hydrogels using F-ANOVA. * = Statistically significant (p < 0.05).

2.3.3 Cell Behaviour in Collagen and HA-T Gels

2.3.3.1 Collagen Gels

To investigate the effect of different collagen sources on cell realignment a contraction assay was carried out using F7 and HDFa cells which are commonly used in such studies. The gels where compared to a control of cells embedded in rat tail collagen which contained 10% BSA to inhibit gel contraction. Both AS bovine and AS rat tail collagen caused more than 50% contraction (Table 8) after 24 h. Cells embedded in PS bovine collagen failed to show any contraction and the gel did not appear to maintain its shape well. The lack of telopeptides (in PS collagen) affected cell realignment (collagen contraction) and gel integrity.
Table 8. The effect of telopeptides on collagen contraction using a contraction assay. HDFa and F7 cells were embedded in a collagen gel (5 mg/mL) for 24 h at 37°C and 5% CO₂. The corresponding p values were calculated by comparing the sample values to the values of the control gels using ORM-ANOVA at n=3.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Collagen type</th>
<th>Contraction (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDFa</td>
<td>PS bovine corium</td>
<td>3</td>
<td>0.141</td>
</tr>
<tr>
<td>F7</td>
<td>PS bovine corium</td>
<td>2.5</td>
<td>0.072</td>
</tr>
<tr>
<td>HDFa</td>
<td>AS bovine corium</td>
<td>55.1</td>
<td>0.017</td>
</tr>
<tr>
<td>F7</td>
<td>AS bovine corium</td>
<td>66.2</td>
<td>0.023</td>
</tr>
<tr>
<td>HDFa</td>
<td>AS rat-tail</td>
<td>60.7</td>
<td>0.033</td>
</tr>
<tr>
<td>F7</td>
<td>AS rat-tail</td>
<td>58.3</td>
<td>0.019</td>
</tr>
</tbody>
</table>

The effect of cell growth/culture in a 3D environment (cells embedded in a collagen gel) was compared to that in a 2D environment (cells grown on a flat surface in culture plates). The effect of embedding HDFa cells in different collagen preparations (3D cultures) on cell viability was also analysed. The cell viability in all three of the collagen type I preparations was lower than cells grown in a 2D culture (Figure 23). The collagen source and lack of telopeptides had no significant on effect (p= 0.382) on cell viability in a 3D microenvironment. After 24 h the cell viability was approximately 70% for the HDFa cell embedded in different collagen gels.
Figure 23. The effect of HDFa cells embedded in 3D cultures of collagen gels on cell viability after a 24 h incubation at 37°C and 5% CO₂. Control = cells grown in 2D without collagen, AS = acid soluble collagen and PS = pepsin soluble collagen. The values represent the mean ±SE at n=3. p values > 0.05 when the different conditions were compared using ORM-ANOVA. N/S = Not statistically significant (p > 0.05).

2.3.3.2 3D Cell Cultures of HA-T Gels

The cell viability of HDFa cells grown in various HA-T gels was investigated to study whether such gels could be used alone or in combination with collagen gels to create 3D cell constructs. There was a significant reduction in cell viability of control (cells grown in rat tail collagen gel) after 24 h (Figure 24). The cell viability level was dependent on the HA-T concentration (p= 0.026). The percentage viability was around 20% at 5 mg/mL of HA-T gel and dropped to around 15% at 2.5 mg/mL. Tyramine substitution levels had no significant effect on cell viability at both 2.5 mg/mL (p= 0.238) and 5 mg/mL (p= 0.404) of HA-T gels.
Figure 24. The effect of in-situ crosslinking of HA-T hydrogels using H$_2$O$_2$ to create 3D culture of HDFa cells vs HDFa cells grown in AS rat-tail collagen (control) on cell viability. Tyramine substitution levels used were 1-5% at 2.5 mg/mL and 5 mg/mL biopolymer concentration. 3D cell cultures were incubated at 37°C and 5% CO$_2$ for 24 h. The values represent the mean ±SE at n=3. p values > 0.05 when the different substitution levels were compared at the same biopolymer concentration using ORM-ANOVA. p values <0.001 when different concentrations were compared using ORM-ANOVA. N/S = Not statistically significant (p > 0.05). * = Statistically significant (p < 0.05).

A H$_2$O$_2$ concentration-response study was conducted to determine whether the H$_2$O$_2$ concentration required for the crosslinking was the result of the toxicity seen in those 3D cultures. In addition to HDFa cells, F7 cells were utilised to find out whether a secondary (immortalised) cell line has a different sensitivity to the peroxide. H$_2$O$_2$ showed a dose dependent effect on cell viability (Figure 25) which increased with peroxide concentration (p< 0.001, using within subject F-ANOVA). It was shown that the required concentration of H$_2$O$_2$ (1.2 µM) to crosslink the gel resulted in a very low cell viability in both types of cells resulting 0 % and 1% cell viability of control for HDFa and F7 cells, respectively. HDFa cells were more susceptible to the peroxide when compared with F7 cells (p< 0.001). The cell viability of F7 cells at 0.029 µM of H$_2$O$_2$ was 97.3% but was 58.0% for HDFa cells.
Figure 25. The dose response curve showing the effect of different concentrations of H₂O₂ on HDFa and F7 cells after 24h of co-incubation at 37°C and 5% CO₂. The values represent the mean ±SE at n=3. *p< 0.001 when the two dose response curves were analysed using F-ANOVA. * = Statistically significant (p < 0.05).

2.3.4 Artificial Vitreous

To establish a proof of concept for the preparation of an artificial vitreous, type I collagen was utilised because type II collagen is expensive [3]. As described earlier collagen type I contains two α2(I) and one α1(I) polypeptide chains and collagen type II contains three α(II) polypeptide chains [3], [16]. The collagen type II amino acid content is very similar to collagen type I [164]. For example, the amino acids known to be involved in the photochemical crosslinking: threonine, tyrosine, hydroxyproline and histidine [66] are slightly higher in content in collagen type II than collagen type I (Table 9). Moreover, photochemical crosslinking of collagen type II has been described in earlier studies using riboflavin and blue light [165], [166].

Collagen and HA were chemically crosslinked to investigate whether this approach could be used for an artificial vitreous preparation. A commercially available HA-T was utilised to allow crosslinking with collagen. HA-T with tyramine substitution levels of 1, 3 and 5% and a molecular weight (Mw) of HA of 870 kDa which is much lower than in the native VH (~5 MDa) were used in these experiments. The viscosity of a polymer is directly
proportional to the Mw of the polymer. Hence, HA-T concentrations used in this study were higher than the HA concentration in the native VH (400 µg/mL) [47].

**Table 9.** The % content of amino acids in collagen type I and II which are involved in photochemical crosslinking of collagen. Data extracted from (Bondarenko and Kovalenko 2012; Chattopadhyay and Raines 2014)[5],[164].

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Collagen type II (% content)</th>
<th>Collagen type I (% content)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine</td>
<td>0.8</td>
<td>0.54</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>9.5</td>
<td>10.32</td>
</tr>
<tr>
<td>Threonine</td>
<td>2.97</td>
<td>1.70</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.70</td>
<td>0.44</td>
</tr>
</tbody>
</table>

**2.3.4.1 Carbodiimide Mediated Crosslinking**

A carbodiimide coupling reaction was used to crosslink collagen with HA (Figure 26A) [167]. Out of the two crosslinking methods used only method 1 resulted in a crosslinked hydrogel as observed by the changes to the appearance and stiffness of the mixture (Figure 26). The mixture changed from a watery consistency (Figure 26.B) to a stiff gel (Figure 26.C) after the reaction. The mixture in method 2 showed no signs of crosslinking in FTIR (lack of an amide stretch). The crosslinked hydrogel prepared from method 1 showed an amide stretch in the FTIR spectra (Figure 27, next page). The N-H stretch (amide) occurred at around 3300 cm\(^{-1}\) in the crosslinked gel and was a sign of crosslinking.
Figure 26. Carbodiimide coupling reaction mechanism between collagen and Hyaluronic acid. Carbodiimide crosslinking the collagen-HA mixture (A) before and (B) after crosslinking using method 1.

Figure 27. The change in FT-IR spectra after crosslinking of collagen and HA separately and the cross-linked mixture.
The viscosity of the crosslinked gel (method 1) was also determined and compared to the published viscosity of the product to the viscosity of the native VH where the data was extracted from Silva et al\textsuperscript{[168]}. The viscosity of the HA-Collagen gel was significantly higher than that of the native vitreous (Figure 28). The gel was also shear thinning which is also a characteristic of natural VH.

![Figure 28](image_url)

**Figure 28.** The viscosity of artificial vitreous humour (VH) (crosslinked HA and collagen) and the native VH (data extracted from Silva, Alves, and Oliveira 2017 (24)). The HA-collagen gel values represent the mean ±SE at n=3.

### 2.3.4.2 Photochemical Crosslinking

Photochemical crosslinking was utilised to prepare the vitreous substitute as it may allow for in-situ crosslinking and therefore crosslinking after injection\textsuperscript{[66]}. The Mw HA-T (~870 kDa) used in this study was lower than that in the native VH (~5 MDa)\textsuperscript{[49]}. To account for that, higher concentrations (> 400 µg/mL of HA) of HA-T polymers with different levels of tyramine substitution were used to attempt to match the viscosity profile of the native VH\textsuperscript{[47]}.

HA-T could be successfully crosslinked at (2-5 mg/mL). A gel of HA-T:Col (4:1, 2.5 mg/mL:0.625 mg/mL) was crosslinked successfully (Figure 29) to prepare the vitreous substitute. Visibly the HA-T: Col gel was stiffer than a HA-T gel at 5 mg/mL HA-T. Solutions of PS collagen were also successfully crosslinked. The yellow colour of riboflavin was also visible which can be
removed by washing. The controls comprised the macromolecules without riboflavin remained solutions after illumination.

**Figure 29.** Different mixtures of photochemically crosslinked HA-T and HA-T:Col using 25 µg/mL riboflavin. 1) HA_T 2.5 mg/mL, 2) HA-T:Col (4:1) 2.5 mg/mL: 0.625 mg/mL and 3) HA-T 5 mg/mL.

Different concentrations of collagen and HA-T (1-5% tyramine) solutions were illuminated for 15-60 min to figure out what concentration crosslinking occurs (Table 10). The illumination time was 15, 30 and 60 min at 4 and 8 mW/cm² using both 25 and 50 µg/mL riboflavin. The same results were observed for the different variations in the crosslinking conditions. At 2 mg/mL a gel formed for both collagen and HA-T (1, 3 and 5% tyramine) solutions but did not maintain its integrity. At 2.5 mg/mL the formed gel maintained its integrity and was utilised in subsequent studies.
Table 10. The effect of biopolymer concentration on the photochemical crosslinking of collagen and HA-T. The presence (+) of crosslinking (gel formation) and the absence (-) of crosslinking (solution) was visually confirmed. At 15-60 min crosslinking time, 4 and 8 mW/cm² intensity, an ambient temperature and 25-50 µg/mL riboflavin and at n=3.

<table>
<thead>
<tr>
<th>Macromolecules</th>
<th>Concentration (mg/mL)</th>
<th>Crosslinking (+/-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA-T at 1%, 3% and 5% Tyramine</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td>PS bovine corium collagen</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>-</td>
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<tr>
<td></td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>+</td>
</tr>
</tbody>
</table>

The effect of the solution thickness on crosslinking was investigated (Table 11). The same profile was observed for solutions of collagen HA-T (1,3 and 5% tyramine). The same results were observed for different crosslinking conditions: 15, 30- and 60-min crosslinking time at 4 and 8 mW/cm² in 25 and 50 µg/mL riboflavin. The thickness limit for crosslinking was 2.62 mm (500 µL in a 24 well-plate). The control which was a solution that lacked riboflavin or HA-T did not become crosslinked and remained a solution (visually confirmed) with no change to its colour or appearance. The conclusion was based on the observation that part of the mixture remained liquid and the upper layer formed a gel. The solution volume was kept at 500 µL in subsequent studies.
Table 11. The effect of mixture volume/thickness on the photochemical crosslinking of collagen and HA-T. The presence (+/ gel) of crosslinking and the lack (-/ solution) of crosslinking was visually confirmed. At 15-60 min crosslinking time, an ambient temperature and 4 and 8 mW/cm² intensity and at n=3.

<table>
<thead>
<tr>
<th>Macromolecules</th>
<th>Volume (µL)/ Thickness (mm)</th>
<th>Crosslinking (±)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA-T at 1%, 3% and 5% Tyramine</td>
<td>125/ 0.65</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>250/ 1.31</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>500/ 2.62</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>750/ 3.92</td>
<td>-</td>
</tr>
<tr>
<td>PS bovine corium collagen</td>
<td>125/ 0.65</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>250/ 1.31</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>500/ 2.62</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>750/ 3.92</td>
<td>-</td>
</tr>
</tbody>
</table>

2.3.4.3 Photochemical Crosslinking in the Deep Red Spectrum

To study whether a thicker solution (HA-T and collagen) can be crosslinked, AlPcS2a was used as photoinitiator which is activated at 660 nm. At this wavelength light penetrates more deeply into the solution [169]. No crosslinking occurred (Table 12) at the different concentrations of AlPcS2a used (50-1000 µg/mL) in both 2.5 and 5 mg/mL solutions of the macromolecules. The same outcome was observed for different crosslink conditions: 15, 30- and 60-min exposure time at 4 and 8 mW/cm². There was the same observation for both collagen and the various HA-T preparations. All of the mixtures remained as a liquid at the end of the reaction. A positive control was prepared with riboflavin as a photoinitiator. The gel fully formed at 2.5 mg/mL of HA-T at 4 mW/cm² and these parameters were therefore chosen for subsequent analysis of crosslinking with collagen (1:4, Col:HA-T). A 30 min crosslinking time was carried in most studies as it was also used by Donnelly et al [67].
Table 12. The effect of various concentrations of AlPcS2a on the photochemical crosslinking of HA-T and collagen. The presence (+/ gel) of crosslinking and the lack (-/ solution) of crosslinking was visually confirmed. At 15-60 min crosslinking time, at an ambient temperature, and 4 and 8 mW/cm² intensity and at n=3.

<table>
<thead>
<tr>
<th>Macromolecules</th>
<th>Macromolecule concentration (mg/mL)</th>
<th>AIPcS2a concentration (µg/mL)</th>
<th>Crosslinking (±)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA-T at 1%, 3% and 5% Tyramine</td>
<td>2.5/ 5</td>
<td>50</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>250</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1000</td>
<td>-</td>
</tr>
<tr>
<td>PS bovine corium collagen</td>
<td>2.5/ 5</td>
<td>50</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>250</td>
<td>-</td>
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<td></td>
<td>500</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1000</td>
<td>-</td>
</tr>
</tbody>
</table>

2.3.4.4 Rheological Characterisation

As the 2.5 mg/mL biopolymer concentration was the lowest concentration at which the gel fully formed it was used in the rheological studies and to optimise the photochemical crosslinking process. The 2.5 mg/mL HA-T was used to figure out the ideal tyramine substitution level and to optimise the crosslinking time and riboflavin concentration. The 2.5 mg/mL HA-T concentration was used to prepare the vitreous substitute. The Col:HA-T hydrogel was prepared at 0.675 mg/mL:2.5 mg/mL: (1:4) as a vitreous substitute.

The viscosity of a 2.5 mg/mL HA-T gel at 5% tyramine (Figure 30) crosslinked for 15-60 min was analysed to establish the ideal crosslinking time. The crosslinking time had no significant effect (p= 0.246) on viscosity. The hydrogels shear thinned with the increase in shear rate (p< 0.001, within subject F-ANOVA). The viscosity dropped from ~100 Pa at 0.1 s⁻¹ to ~1 Pa at 100 s⁻¹.
Figure 30. The effect of photochemical crosslinking time on the viscosity profile of the HA-T gel. Crosslinking was at 2.5 mg/mL HA-T, 5% tyramine, at 4mW/cm² illumination intensity and at an ambient temperature. The values represent the mean ±SE and at n=3. p= 0.246 when the different crosslinking time profiles were compared using F-ANOVA. N/S = Not statistically significant (p > 0.05).

The effect of riboflavin 5’-phosphate concentration (25 and 50 µg/mL) was investigated in terms of its influence on the viscosity of photochemically crosslinked HA-T gels. The riboflavin concentration did not have a significant effect (p= 0.348) on gel viscosity (Figure 31). The gels shear thinned (the viscosity decreased) with the increase in shear rate. The 25 µg/mL riboflavin was used in subsequent studies. The effect of tyramine substitution levels on the viscosity of a 2.5 mg/mL of HA-T gel was also investigated (Figure 32). Tyramine substitution level (1, 3 and 5%) did not have a significant effect (p= 0.174) on the viscosity after crosslinking. Subsequent crosslinking of collagen and HA-T could therefore be carried out using 25 µg/mL of riboflavin and at any tyramine substitution level.
Figure 31. The effect of riboflavin concentration on the HA-T gel viscosity profile. A 2.5 mg/mL HA-T mixture at 5% tyramine in 25 and 50 μg/mL riboflavin was crosslinked for 30 min, at 4 mW/cm² illumination intensity and at an ambient temperature. The values represent the mean ±SE and at n=3. p=0.348 when the two riboflavin concentration profiles were analysed using F-ANOVA. N/S = Not statistically significant (p > 0.05).

Figure 32. The effect of percentage of tyramine substitution (1-5%) on the HA-T gel viscosity profile. A 2.5 mg/mL of HA-T mixture was crosslinked for 30 min at 4 mW/cm² illumination intensity and at an ambient temperature. The values represent the mean ±SE and at n=3. p=0.174 when the three substitution profiles were analysed using F-ANOVA. N/S = Not statistically significant (p > 0.05).
Finally, the effect of increasing the concentration of HA-T (5% tyramine substitution) was analysed (Figure 33) to investigate whether viscosity after crosslinking was concentration dependent in the HA-T. The viscosity of HA-T increased with concentration of HA-T from 2.5 to 5 mg/mL (at 5 % tyramine). Viscosity increased from approximately 100 to 300 Pas for 2.5 and 5 mg/mL respectively (p= 0.004). The viscosity of 5 mg/mL HA-T was 3 (± 0.4) times higher than the 2.5 mg/mL mixture at all the shear rates. Both mixtures were crosslinked using the same parameters (30 min and 4 mW/cm² intensity) and showed a similar shear thinning profile. At a 100 1/s shear rate, the viscosity of the 5 mg/mL composition is double that of 2.5 mg/ due to increase in crosslinking density.

Figure 33. The change in the gel viscosity profile with increase in HA-T concentration. The 2.5 mg/mL and 5 mg/mL HA-T mixtures at 5% tyramine were crosslinked for 30 min at 4 mW/cm² and at an ambient temperature. The values represent the mean ± SE and at n=3 and p=0.004 when two concentration profiles were compared using F-ANOVA. * = Statistically significant (p < 0.05).

A candidate artificial vitreous substitute was prepared at 2.5 mg/mL HA-T and 0.625 mg/mL of collagen (1:4). Lower HA-T concentrations were not used as the gel did not fully form at 2 mg/mL. The crosslinking time of 15-60 min did not affect the viscosity but 30 min crosslinking was chosen as it was also used by Donnelly et al for crosslinking HA-T [67]. An illumination intensity
of 4 mW/cm² was used as it would potentially introduce a smaller risk of toxicity than the 8 mW/cm² in-vivo and has been used to crosslink collagen in experimental studies [170]. Compared to a HA-T only gel the viscosity of HA-T collagen increased (Figure 34). The viscosity of the Col: HA-T gel (artificial vitreous) was almost double that of HA-T only gel (p <0.001). Both hydrogels shear thinned significantly and in the same manner to a viscosity of ~1 Pa at 100 s⁻¹. The crosslinking of collagen and HA-T resulted in a dramatic increase in viscosity indicating the HA and collagen were successfully crosslinked to prepare the hydrogel based artificial vitreous.

![Figure 34](image)

**Figure 34.** The change in the viscosity profile of the Col: HA-T vitreous hydrogel compared to a HA-T hydrogel. The mixtures of 2.5 mg/mL HA-T and Col: HA-T (0.625 mg/mL: 2.5 mg/mL) were crosslinked for 30 min at 4 mW/cm² and at ambient temperature. The values represent the mean ±SE and at n=3. p < 0.001 when two hydrogel profiles were compared using F-ANOVA. * = Statistically significant (p < 0.05).

### 2.3.4.5 Biocompatibility of In-Situ Crosslinking

As riboflavin is already used clinically to crosslink collagen in the cornea, in-situ crosslinking of the vitreous substitute was also investigated by using an in-vitro cell viability assay. ARPE-19 cells were used in the study as being retinal cell line made them biorelevant. ARPE-19 cells exposed to blue light (for 15-30 min at 470 nm and 4 mW/cm²) did not display signs of reduction in cell viability (p= 0.384) after 24 h (Figure 35). The cell viability was above 90% of
the control which was cells grown in a 2D environment without exposure to the blue light.

Figure 35. The ARPE-19 cell viability after exposure to blue light. A 15 and 30 min of exposure to blue light (470 nm at an illumination intensity of 4 mW/cm²) and a subsequent 24 h incubation of ARPE-19 cells at 37°C and 5% CO₂. The values represent the mean ±SE and at n=3. p= 0.384 when the two exposure times were compared using ORM-ANOVA. N/S = Not statistically significant (p > 0.05).

To control for any potential effects on cell viability of the HA-T, collagen and riboflavin in the absence of light, cell viability was determined without blue light illumination. After a 24 incubation the cell viability (Figure 36) remained high (~90%) for the collagen and HA-T solutions. All the different HA-T solutions (1, 3 and 5% tyramine) were incubated with ARPE-19 cells at 5 mg/mL, apart from the HA-T:Col which was incubated at 2.5 mg/mL HA-T (5% tyramine) and 0.625 mg/mL collagen. There was no significant difference (p= 0.321) between the cell viability in the different conditions. The control was ARPE-19 cells grown without exposure to these materials or to blue light.
The ARPE-19 cell viability after a 24 h incubation in HA-T and Col: HA-T solutions in 25 µg/mL riboflavin. The solutions were 5mg/mL HA-T (1-5% tyramine) and HA-T: Col (2.5 mg/mL:0.625 mg/mL) incubated at 37°C and 5% CO₂. The values represent the mean ±SE and at n=3. p=0.321 when the four different biopolymers were analysed using ORM-ANOVA. **N/S** = Not statistically significant (p > 0.05).

The effect of a 15 min photolytic crosslinking *in-situ* was tested in of HA-T and HA-T:Col solution (Figure 26). After 24h the cell viability of ARPE-19 cells exposed to blue light was high (> 90%). There was a high reduction in cell viability when crosslinking was carried out using 25 µg/mL riboflavin for 15 min, at 4 mW/cm². For HA-T gels (1-5% tyramine) the cell viability was ~20% and ~30% in 2.5 and 5 mg/mL polymer concentration. The polymer concentration had a significant effect on cell viability (p <0.001). Tyramine substitution levels did not have a significant effect on cell viability for both 2.5 mg/mL (p= 0.238) and 5 mg/mL (p= 0.404) gels. The Col:HA-T (0.625 mg/mL collagen and 2.5 mg/mL HA-T) gel displayed a similar cell viability (p= 0.08) to the 5 mg/mL sample. This indicated that a higher gel mechanical stiffness resulted in improved cell viability. Molecular movement is lower in a stiffer gel and hence less exposure of the cells to toxic molecules created during the reaction.

A 30 min *in-situ* photolytic crosslinking under the same crosslinking conditions as the 15 min experiment resulted in lower cell viability (Figure 38). The crosslinking time had a significant effect on cell viability (p <0.001).
Tyramine substitution levels did not have a significant effect on cell viability in 2.5 mg/mL (p= 0.692) and 5 mg/mL (p= 0.258) HA-T gels. The cell viability increased with the increase in polymer concentration (p <0.001). The Col:HA-T:Col gel showed a cell of 11% of control after 24 h and was higher than HA-T gels at 2.5 mg/mL (p< 0.001).

Figure 37. The cell viability after a 15 min in-situ crosslinking in ARPE-19 and a subsequent 24 h incubation. Crosslinking was conducted at a 4mW/cm², using 2.5-5 mg/mL HA-T (1-5% tyramine) and HA-T:Col (2.5 mg/mL:0.625 mg/mL). The values represent the mean ±SE and at n=3. All p values <0.001 when the different HA-T preparations were compared with each other and with the HA-T:Col preparation using ORM-ANOVA. * = Statistically significant (p < 0.05).
Figure 38. The cell viability after a 30 min in-situ crosslinking in ARPE-19 and a subsequent 24 h incubation. Crosslinking was conducted at a 4mW/cm², using 2.5-5 mg/mL HA-T (1-5% tyramine) and HA-T:Col (2.5 mg/mL:0.625 mg/mL). The values represent the mean ±SE and at n=3. All p values <0.001 when the different HA-T preparations were compared with each other and with the HA-T:Col preparation using ORM-ANOVA. * = Statistically significant (p < 0.05).

2.3.5 Summary of Results

AS rat tail collagen gels had the highest mechanical stiffness and the PS based collagen gels had the lowest mechanical stiffness. The HDFa embedded gels from different collagen sources (with and without telopeptides) had similar cell viability. The cell viability of HDFa cells in 3D cultures was lower than in 2D cultures. Atelocollagen (PS) failed to show signs of contraction (cell realignment). Enzymatically crosslinked HA-T gels had a higher mechanical strength than collagen gels but caused a significant reduction in the cell viability of HDFa cells embedded inside them due to the H₂O₂.

Preparation of the artificial vitreous was conducted by carbodiimide mediated coupling to crosslink collagen and HA. Photochemical crosslinking was also successfully used to prepare an artificial vitreous of crosslinked collagen and HA-T with tuneable mechanical properties and a shear thinning profile seen with natural vitreous. It can potentially be used for in-vitro and in-vivo models. The ideal crosslinking conditions were 2.5 mg/mL HA-T in 0.675 mg/mL collagen solution, crosslinked using 25 µg/mL riboflavin for 30 min at 4
mW/cm². AlPcS2a was unable to induce gel photo-induced crosslinking. In-situ crosslinking resulted in significant drop in cell viability due to the direct exposure to high levels of ROS.

2.4 Discussion

The absence of telopeptides resulted in mechanically weaker collagen hydrogels (Figure 18). Collagen telopeptides play a major role in stabilising mature collagen fibrils [3], [171]. Fibril formation is an entropy-driven process where self-assembly results in burying surface-exposed hydrophobic residues within the fibril, thereby increasing entropy in the solvent. The fibril formation therefore occurs in both AS and PS collagens. The presence of telopeptides allows AS collagen to assemble in a more organised manner where the fibrils are more regularly aligned increasing the mechanical strength of the gel in the process [172].

Holmes et al also identified that atelocollagen type I has a lower shrinkage temperature ($T_s$) than telocollagen. $T_s$ is a measure of intermolecular interactions where a higher $T_s$ indicates the presence of a higher degree of intermolecular interactions. The reduction in $T_s$ is a result of the lack aldol/covalent cross-links occurring between the C- and N-terminus telopeptides to the adjacent molecule) in atelocollagen due the lack of telopeptides [12].

Porcine and bovine collagen type I are biocompatible and are used clinically [31], [78]. As described in the introduction the presence of telopeptides results in an increase in antigenicity to collagen in some patient [29]. Atelocollagen is a more clinically attractive biomaterial for use in biomedical applications due to its lower immunogenicity risk [29].

Collagen type I from bovine corium had a lower mechanical stiffness than rat-tail collagen (Figure 18). Collagen type I in tendons has a much higher mechanical stiffness than collagen in the corium (skin). This due to the higher degree of fibril alignment along the collagen in tendons [173], [174]. In humans the 3-hydroxylation of a C-terminal motif in type I collagen is unique to the tendon and is absent from collagen type I in skin and bone. This can contribute to the difference in the mechanical properties between the same type of collagen in the different tissues [3], [175].
An objective of this work was to establish whether the lack of telopeptides in PS bovine collagen affects the biological behaviour of collagen in 3D cultures. Collagen contraction is an indication of cell alignment in tissues and the ECM which is essential in human biological processes such as wound healing where collagen plays a major role. PS bovine collagen 3D cell cultures did not show any signs of gel contraction (Table 8). This can be due to the slow gel formation (25 min) in PS collagen compared to the AS collagen (15 min). This meant that cells settled down at the bottom of the gel which impaired their ability to align the collagen fibrils as they were not well distributed in the gel. The lack of telopeptides also resulted in the gel with very weak integrity which might have affected the cell attachment to the collagen fibrils. Woodley et al showed that lack of telopeptides in collagen type I lattices significantly inhibited collagen contraction in 3D cultures.

Enzymatically crosslinked HA-T gels utilising HA with different tyramine substitutions from 1, 3 and 5% had similar mechanical strength profiles (Figure 20). This can be caused by the fast gel formation (< 5 s) upon addition of the H₂O₂, not giving enough time for the peroxide to mix with the gel homogenously. Certain areas inside the gel may not become crosslinked which can affect the mechanical strength. The HRP enzyme probably generated oxygen radicals from the peroxide of H₂O₂ which rapidly react with everything in solution including the HA-T. These radicals probably cannot permeate into the HA-T to cause a reaction. Once many of the surface tyramine undergo reaction, the it is possible that further reaction was precluded. The mechanical stiffness increased when the polymer concentration was doubled (Figure 21). This change could be due to the increase in the polymer content of the gel which in turn increased the number of crosslinks.

The formation of 3D network in the HA-T hydrogels through covalent bonds between the aromatic rings in the tyramine molecules resulted in gels that are more resistant to deformation. The crosslinks act to hold the 3D structure of the gel together and protect it from mechanical deformation. HA-T hydrogels were mechanically stronger than collagen hydrogels (Figure 22). HA can form a larger number of hydrogen bonds than collagen and binds water molecules a1000× its own weight. HA-T hydrogels may contain less
unbound water molecules which led to their mechanical stiffness not increasing to the same degree as collagen hydrogels, as the oscillation frequency increased \[46\], \[177\], \[178\].

The cell viability in 3D cultures of collagen gels was significantly lower (around 65%) than those grown in 2D cultures (Figure 23) Several factors may be involved such as cells grown in a 2D environment are normally flatter and more stretched than in an in-vivo environment \[179\]. The difference in cell morphology also influences many cellular processes including cell proliferation, differentiation, and gene expression. Differences in cell migration exist between a 2D surface and a 3D environment. In a 2D environment the cell is confined to one plane in 2D and encounters little to no resistance to migration from a surrounding ECM (or collagen hydrogel). These differences may have led to the decreased cell viability in 3D cultures compared to 2D cultures \[180\]–\[182\].

To investigate the possibility of using HA-T for 3D cell cultures for tissue engineering applications, the hydrogel was used to grow F7 and HDFa cells which are commonly used in-vitro experiments. 3D cultures of HA-T hydrogels showed a low cell viability (Figure 24) which decreased with the decrease in concentration. The use of H\textsubscript{2}O\textsubscript{2} at the recommended concentration resulted in low cell viability in both F7 and HDFa cells due to the oxidative activity of H\textsubscript{2}O\textsubscript{2} (Figure 25). Excess ROS generated by H\textsubscript{2}O\textsubscript{2} can overwhelm a cell's antioxidant scavenging capacity, causing oxidative damage to DNA, lipids, and proteins causing cellular damage \[183\]. Interestingly, cytotoxicity decreased with increased HA-T concentration which could be due to the higher gel stiffness at 5 mg/mL limiting diffusion of the peroxide and ROS (generated by the reaction). Thus, fewer cells were exposed to the damaging radicals during the gelling process and before the media addition which would dilute the ROS.

Although ATP based assays such as CellTiter-Glo are widely used to measure cell viability they cannot distinguish between cell cycle inhibition and cell death. A lactate dehydrogenase (LDH) assay could therefore be used in addition to the CellTiter-Glo assay to measure the levels of cytotoxicity and allow for further improvement of analysis of the studies in this thesis \[184\]. LDH is a cytosolic enzyme which is released into the growth medium upon loss of integrity of cell membranes and is therefore used to measure the levels of
cytotoxicity \[185\]. Moreover, the type of cell death in the studies could be further analysed by using a necrosis-apoptosis assay \[186\]. Apoptosis is a form of programmed cell death that occurs in multicellular organisms. Necrosis is an unregulated, accidental cell death which can be caused by nonspecific, or non-physiological stress inducers \[187\].

In contrast to Darr and Calabro reported that 3D cultures of HA-T gels showed excellent chondrocyte cell viability. This can be due to the washing step (with PBS for 5 min) immediately after the crosslinking process to remove any unreactive \(\text{H}_2\text{O}_2\) and ROS by-products that can cause further damage to cells. A 20× higher cell seeding density was used which meant that the total exposure of to \(\text{H}_2\text{O}_2\) per cells was 20x less than that in our study. This difference may have contributed to the difference in cell viability \[68\].

Although HA and collagen could be crosslinked by carbodiimide mediated coupling (Figure 26) this crosslinking strategy was not ideal for forming an artificial VH. The reaction involves many side reactions including iso-urea formation which results in HA or collagen functionalisation with a non-reactive carbodiimide derived moiety to give heterogenous by-products which are to purify \[83\], \[167\].

Photochemical crosslinking of HA-T and collagen type using riboflavin was successfully used to prepare the hydrogel based artificial vitreous. Crosslinking ensured long term stability of the hydrogel network without the two components separating with time. AlPcS2a was investigated as a potential crosslinker because blue light does not penetrate more than few mm (Table 11). The red-light wavelength can penetrate deeper and was therefore a more ideal candidate. Unfortunately, no crosslinking was achieved (Table 12) when the AlPcS2a crosslinker was used. This can be related to the fact that AlPcS2a is an amphiphilic molecules which can affect its ability to form ROS in hydrogel environment \[188\].

The viscosity of the crosslinked artificial vitreous (Figure 34) was much higher than that of the native VH which can be due the concentration of both collagen and HA being higher. The concentration could not be dropped below 2.5 mg/mL oh HA-T as the gel could not form fully form. The Mw of HA in the vitreous is around 5 MDa \[47\] which is higher than that of HA-T (870 kDa).
Donnelly et al showed higher mechanical stiffness values when they crosslinked HA-T at the same riboflavin concentrations. They used 10 mW/cm² at 365 nm and carried out crosslinking at higher polymer concentrations (10-30 mg/mL). These differences could have led to the difference in the mechanical stiffness differences [67].

Small angle X-ray scattering (SAXS) can be utilised in addition to the viscosity measurements to further analyse the crosslinked gels. SAXS measures the intensities of X-rays scattered by a sample as a function of the scattering angle where measurements are made at very small angles. The resulting scatter gives an indication of the gel structure and morphology [189].

Tytgat et al photochemically crosslinked methacrylate derived gelatine (degraded collagen) using the Lithium (2,4,6-trimethylbenzoyl) phenylphosphinate photoinitiator to produce 3D cell cultures. The results showed a good cell viability which is promising. It can be that compared to this study the cell density was 10x higher and the crosslinking time was only 10 min [190]. It can also be the result the photo initiator which potentially generates less ROS therefore resulting in less toxicity.

The effect of crosslinking in-situ on cell viability was also due to the creation of the ROS which the cells get exposed for 15-30 min (Figure 37 and Figure 38) without any dilution or removal of those species. An increase in macromolecule concentration reduced toxicity as the higher degree of crosslinking mean that the less ROS was in contact with the cells.

Riboflavin is clinically used at higher concentrations than in this study to crosslink collagen in the cornea without any apparent toxicity. Clinically in the treatment of keratoconus the stromal surface of the cornea is soaked with 1 mg/mL of riboflavin solution. To photochemically crosslink the collagen, ultraviolet light (370 nm at 3 mW/cm²) is applied for 30 min [191]. The method used in this study to test the biocompatibility might be the issue as it does not allow for the removal of any toxic by-products such as the reactive oxygen species generated during the reaction. For example, in-vivo the flow of the aqueous humour ensures the continuous the removal of such compounds from the area [39]. Microfluidics-based devices might potentially provide more biomimetic environment for studying the biocompatibility of in-situ crosslinking.
as the continuous flow of media would allow for the removal of the ROS generated during the reaction.\textsuperscript{192}

In order to favour the clinical application of collagen gel formulations containing therapeutic agents (e.g. anti-scarring drugs), an \textit{in-situ} gelling hydrogel \textit{without} the use of chemical crosslinking might also be worthy of further exploration.
Chapter 3: Preparation and Optimisation of *In-Situ* Polymerisable Collagen
3. Preparation and Optimisation of *in-situ* Polymerisable Collagen

3.1 Introduction

Hydrogels may potentially have utility as a drug and cellular delivery platforms, but the high-water content and large pore sizes of most hydrogels often results in a rapid release of therapeutics. The administration of hydrogels as a dosage form is also an issue, although some hydrogels are sufficiently deformable and may shear thin (e.g. HA) to be injectable. Issues with biocompatibility, unpredictable biodegradability and weak mechanical properties still plague many of the experimental hydrogels [94], [193].

A hydrogel prepared from an *in-situ* polymerisable collagen (IPC) is a good candidate to investigate as collagen undergoes minimal changes in volume when undergoing gelling [107]. An IPC has been prepared previously for use in tissue augmentation and has been proposed to be used for drug delivery [106], [109]. The IPC was evaluated in phase I and phase II clinical trials for subcutaneous tissue augmentation. The preparation was safe and effective and did not show signs of hypersensitivity after 9 months of monitoring. This indicates that the IPC should not be sensitive or toxic to skin [108], [109]. Sodium ethylenediaminetetraacetic acid (EDTA) is used to stabilise the collagen molecules at pH 7 resulting in a solution which can be injected using a small gauge needle. Upon exposure to a biological environment, the EDTA can diffuse from the collagen molecules allowing it to form fibrils. EDTA is also an effective inhibitor of gelatinases and collagenases and can slow the degradation of collagen *in-vivo* and allow it to carry out its function and might further reduce the risk of any immune response due to collagen degradation [142].

The mechanism of stabilisation by the EDTA has not been closely studied but it is related to the fact that EDTA is a metal chelator. EDTA can chelate various metal ions (*Figure 39*) and is thought to interacts with collagen to form an intermediate state that keeps the collagen molecules apart at pH 7 [105], [107], [108]. It has been shown EDTA binds to the collagen molecules (non-covalently) with some EDTA remaining associated with the collagen fibrils even after fibrillogenesis [194].
Mannitol is a 6-carbon sugar alcohol with 6-hydroxy groups and sucrose is a disaccharide of fructose and glucose. These two polyol compounds can be used to maintain the isotonicity of solutions and have been used to stabilise collagen by increasing thermal stability. Increasing thermal stability allows collagen to remain in a solution at ambient temperature for longer periods than without the presences of these polyol compounds. As already mentioned, fibrillogenesis is a temperature dependent process. Polyois and some saccharides can increase the thermal stability of proteins in solution by stabilising protein tertiary structure, e.g. bovine pancreas ribonuclease \[^{195}\]. Mannitol and sucrose also increase the surface tension of water which is also a contributory factor for protein stabilisation \[^{196}\], \[^{197}\]. Gekko and Koga established that sugars and polyols stabilise fibrous proteins like collagen by strengthening hydrophobic interactions between the non-polar regions of collagen in the solution \[^{198}\].

### 3.1.1 Aims

The aim of the work described in this chapter is to optimise a method for preparing an IPC reproducibly. IPC fabrication is only vaguely described in a patent \[^{105}\]. A method for preparing an IPC gel with tuneable mechanical properties will be explored. Potential formulation methods to exploit the mechanical properties of the gel will also be investigated. Several analytical
techniques (chemical, physical and morphological) will be utilised to characterise the IPCs to determine potential applications of this type of collagen gel.

3.2 Material and Methods

3.2.1 Materials

The following materials were purchased from commercial sources. Acid and pepsin soluble type I bovine collagen (Collagen Solutions, UK), Dialysis Tubing- Visking 12-14 kDa (Medicell Membranes Ltd, UK), 24-well and 96-well plate (Thermo Fisher Scientific, UK), collagenase (GIBCO, US); Hanks Buffer Salt Solution (HBSS) (GIBCO, US), total collagen assay kit (Abcam, ab222942), Pierce BCA protein assay kit (Thermofisher Scientific, US), plate seal (Star Lab, UK). From Sigma-Aldrich, UK: sodium hydroxide (NaOH), sucrose, phosphate buffered saline tablets, D-mannitol, EDTA disodium salt dihydrate, female luer coupler, poly(ethylene glycol) 20,000 Da and sodium azide.

3.2.2 Instrumentation

Sorvall Legend RT+ Centrifuge (Thermo Scientific, US); VirTis AdVantage freeze drier (SP Scientific, US); Spectrmax M2 plate reader (Molecular Devices, US); using Milliosmol Osmometer (Roebling, US); Bohlin Gemini HR Nano rheometer (Malvern, UK); Thermo Quanta 200F scanning electron microscope (FEI, USA).

3.2.3 Methods

3.2.3.1 IPC Preparation and Optimisation

To prepare the IPC solution, a series of dialysis against multiple pH adjusted ethylenediaminetetraacetic acid (EDTA) solutions (pH 5 -7 ± 0.2) was employed to obtain a collagen solution with a pH value of 7.0 ±0.2. The method was adapted from Devore, Brookes, and Byrnes 2014 [105] but was further optimised as very insufficient details of the preparation process were reported. Briefly, an excess of saturated sodium chloride (NaCl, 0.8 M) solution was
added to type I pepsin-soluble (PS) and acid soluble (AS) collagen solution (6 mg/mL) to precipitate the collagen. The white, opaque precipitate was then pelleted by centrifugation at 4600 rpm for 20 min at an ambient temperature. The NaCl solution was discarded and excess NaCl solution was left to drain from the precipitate. The more NaCl solution is removed from the precipitate the higher the collagen concentration will be in the final IPC.

The collagen precipitate was then transferred into a dialysis tubing with a molecular weight cut-off of 12-14 kDa. Dialysis (at 4-8 °C) was conducted against excess 0.1 M acetic acid (at least 20 times the collagen volume) for at least 48 h. The resulting clear, viscous collagen solution was then dialysed against 35 mM EDTA at a starting pH of 5.0 ±0.2 for at least 10-12 h. Subsequent dialysis steps against 35 mM EDTA at pH 5.5, 6.0 and 6.5 were conducted in a stepwise manner for at least 10-12 h each at each pH value. The collagen concentrate was subject to a final dialysis step against 35 mM EDTA containing 3.5% of D-mannitol and 100 mM sucrose pH 7.0 ±0.2. A schematic diagram of the fabrication procedure is illustrated in Figure 40.

![Figure 40](image)

**Figure 40.** A diagram of the perpetration process of IPC involving a series of dialysis in EDTA solutions by increasing the pH incrementally to pH 7.

### 3.2.3.1.1 Reduction of EDTA Concentration

IPC solutions were prepared using both PS and PS type I collagen from bovine corium as described earlier. The final pH change (7 ± 0.2) was conducted using different EDTA concentrations ranging from 10-35 mM EDTA (in100 mM
sucrose and 3.5% mannitol, pH 7.2). After 10-12 h, the gels were observed for the presence of any cloudiness which indicated the fibrillation of collagen. Cloudy/white gels (visually determined) were considered unstable and that EDTA concentration was considered too low to stabilise the collagen.

IPC solutions that seemed stable at the end of dialysis were tested for changes in gelling time. The IPC solutions were pipetted into 1.5 mL centrifuge vials and were stored at 4-8°C, at ambient temperature and at 37°C. Visible changes in the clarity of the sample appearance by the presence of opaque areas in the solution were used as an indication of instability as it is a sign of fibrillogenesis.

### 3.2.3.1.2 Effect of IPC Dilution

An IPC solution was mixed with a solution of 15-35 mM EDTA (including 100 mM sucrose and 3.5% mannitol) at different ratios using the syringe mixing method (as described in section 2.2.3.4.1). The mixtures were mixed for 3 min and the homogeneity and clarity of the mixture was observed at the end of the mixing process. In addition, the mixtures were visually observed for their gelling characteristics. The different proportions of IPC to a solution of different EDTA concentrations was tested as well as IPC to EDTA solution ratio.

### 3.2.3.2 Excipient and Salt Incorporation into the IPC

#### 3.2.3.2.1 Effect of Polymers

Poly(ethylene glycol) (PEG, 20,000 Da) was dissolved in a solution of 35 mM EDTA (including 100 mM sucrose and 3.5% mannitol) at pH 7. Using the syringe mixing method, the solution was mixed with IPC at different ratios and PEG concentrations. The syringe mixing method (section 2.2.3.4.1) involves using two syringes contacted by a female luer connector. The solutions were stored at 4-8°C for 30 days and signs of visible fibrillation were monitored every 7 days by observing any appearance of cloudiness in the solution.

#### 3.2.3.2.2 Effect of Salts

Phosphate buffered saline (PBS) tablets was added to a solution of 35 mM EDTA (in 3.5% mannitol and 100 mM sucrose, at pH 7) to obtain a solution
containing 10 mM PBS. The solution was then mixed with the IPC solution using the syringe mixing method. The solutions were mixed at 1:1-1:4 ratios of IPC: solution. Moreover, 15 mM of sodium azide was prepared in the same solution of EDTA and mixed with the IPC as described earlier.

A portion of each of the solutions was then incubated with a preheated PBS solution (at 1:2 ratio at 37°C) and the gelling time was compared to an IPC solution at the same collagen concentration but that lacked PBS or sodium azide. Solution samples of IPC containing salts were also stored at 4°C for 30 days to monitor for signs of visible fibrillation daily for the first week then weekly, by checking if gel develops any cloudiness.

3.2.3.2.3 Effect of IPC Lyophilisation

IPC solutions with different collagen concentrations (20-45 mg/mL), volumes (50-500 µL) and different EDTA concentrations were pipetted (using a positive displacement pipette) into 1.5mL Eppendorf tubes and lyophilised for 72 h. The freeze-dried pellets were then reconstituted by adding DI water to achieve the same collagen concentration as before lyophilisation. The mixture was then pipetted up and down to mix the solution to dissolve the mixture which was then subsequently centrifuged at 4000 rpm to remove air bubbles created during the mixing process. The cake/pellet was also cut into small pieces prior to reconstitution to test the effect of dried sample size on the reconstitution time of the solution.

3.2.3.3 Quantification of Collagen Concentration

3.2.3.3.1 BCA Assay

The collagen content in IPC was measured using Pierce BCA assay which measures the collagen concentration by indicating the amount of amide bonds in the mixture. Briefly, the albumin standards (25 µg/mL to 2 mg/mL) was prepared in PBS solution (10 mM) at both pH 7.2 and 11. PS bovine corium collagen standards were prepared by diluting the starting material (6 mg/mL) to concentrations also ranging from 25 µg/mL to 2 mg/mL in pH 11.

A standard curve was prepared according to the manufacturer’s protocol. Batches of IPC solutions were then diluted 50 times in pH 11.
Standard curve samples (25 µL, from both albumin and collagen) or unknown sample is pipetted into each well in a 96 well plate. Reagent A and B (200 µL, 50:1 ratio) was then added to the well plates. The plate was shaken on a plate shaker for 30 s and at 250 rpm and then incubation at 37°C for 30 min. The absorbance was then measured at 562 nm using a plate reader.

3.2.3.3.2 Hydroxyproline Assay

The protocol was carried out following the manufacturer’s instructions. Briefly, IPC (100 µL) and a collagen type I standard (100 µL, 3 mg/mL) were separately transferred to cryo-vials and 10 N NaOH (100 µL) was added to each vial and the cap was then securely tightened. The mixture was then hydrolysed at 120°C for 1 h in an oil bath and subsequently cooled on ice before the addition of 10 N HCl (100 µL) to each vial to neutralise the solutions.

The samples were centrifuged at 10,000 rpm for 5 min to remove any insoluble precipitate. The collagen standard was then diluted with DI water to prepare a series of concentrations (0-1 mg/mL) to produce a standard curve. The IPC solutions or samples from degraded collagen were diluted (1:15 to 1:100) to ensure the absorbance values fall within the standard curve. Each test sample (10 µL) and standard curve samples (10 µL) were pipetted into wells in a 96 well plate. The sample hydrolysate and standard curve wells were evaporated to dryness by heating the plate at 65°C on a hotplate.

Oxidation Mix (100 µL) was added to each well and the plate was then incubated for 20 min at an ambient temperature. Developer solution (50 µL) was added to each well and the plate was incubated for 5 min at 37°C. Finally, DMAB Concentrate (50 µL) was added to each well and the plate was incubated at 65°C for 45 min on a hotplate. The plate allowed to reach an ambient temperature and the absorbance was then measured at 560 nm using a plate reader. The hydroxyproline assay standard curve (Appendix, Figure 117) was used to determine sample collagen concentration.

3.2.3.4 Osmolality of IPC Solution

The osmolarity of the IPC solution was measured using an osmometer. An osmometer utilises the freezing point depression of water that occurs due to
the addition of solutes to determine the osmolality. Osmolality is measured as the number of milliosmoles per kilogram (mOsm/kg). The instrument was first calibrated with 100 µL DI water and a 100 µL standard with an osmolarity of 300 milliosmole/kg. After calibration samples of IPC solutions (100 µL) were read in triplicate where each batch (n number) was split into three samples (100 µL/sample) and then read by the osmometer. The remaining batches (n numbers) were analysed in the same manner.

3.2.3.5 In-vitro Collagen Degradation

IPC solutions (200 µL) containing 50 mg/mL collagen were pipetted into 1.5 mL centrifuge vials and prewarmed (37°C) PBS solution (400 µL) was added to the vials which were then incubated at 37°C for 2 h to induce fibrillogenesis (gelling). Then the PBS solution was removed followed by washing with DI water (400 µL). For the control, collagen gel was prepared by neutralising PS bovine corium collagen (6 mg/mL) and then incubating it at 37°C for 25 min. The control contained an equal amount of collagen (1.5 mg) per vial to that of the collagen in IPC samples. After gelling, DI water was added to the control samples.

The DI water was removed and 200 U/mL collagenase (Clostridium histolyticum) solution (400 µL) in Hank’s Balance Salt Solution (HBSS) was added to the vials and incubated at 37°C for 1, 3, and 24 h. At the end of each time point, the liquid in the vials was collected and the collagen concentration subsequently measured by the hydroxyproline assay.

3.2.3.6 Mechanical Characterisation of IPC Gels

IPC gels were prepared to compare the effect of collagen concentration, the mixing process and lyophilisation on the mechanical properties of IPC gels. To compare the effect of collagen concentration on the IPC mechanical properties two IPC batches containing 26 mg/mL and 37 mg/mL collagen (determined by hydroxyproline assay).

The effect of syringe mixing on the IPC mechanical properties was analysed by mixing different IPC solutions using the syringe mixing method for
different lengths of time (2 and 6 min) at a similar rate. These samples were then gelled alongside a control which has not undergone mixing.

The effect of lyophilisation on the mechanical properties was also analysed by reconstituting a freeze-dried IPC sample and then triggering its gelling alongside a control of the same batch which has not been lyophilised. The gel preparation process of these samples involved the addition of the IPC solution (400 µL) to a 24 well-plate. The solution was next allowed to undergo fibrillogenesis (gelling) by adding excess PBS solution (1.2 mL). The plate was then incubated for 2 h at 37°C. The gel mechanical stiffness was measured using a rheometer at 5% strain, 0.1-10 Hz oscillation, a gap size of 900 µm and at 37°C.

3.2.3.7 Morphological Characterisation

3.2.3.7.1 Scanning Electron Microscopy

An IPC solution (10 µL) was gelled by adding DI water (30 µL) and incubating the gel at 37°C for 30 min. Also, another batch of un-gelled (not-fibrilised) IPC was added to an Eppendorf vial. The DI water was removed after gelling, and the sample was then washed with DI water (2x) to remove the EDTA, sucrose and mannitol. The samples were then freeze-dried for 72 h. The lyophilised samples were mounted on gold grids for analysis by scanning electron microscopy to visualise the fibrils in the collagen.

3.2.3.7.2 Cryogenic Transmission Electron Microscopy (Cryo-TEM)

IPC gels with a layer thickness ≤2 µm were prepared to allow the electron beam in TEM to pass through to produce a clear image. Different volumes of IPC and DI water were examined to find the ideal sample preparation process. Briefly, IPC (~40 mg/mL collagen) solution (1 µL) was co-deposited with DI water (4 µL, prewarmed at 37°C) on 3 mm HC grids (EM-Resolutions, UK) which had been glow discharged before the deposition. Glow discharging renders the grid hydrophilic and able to take up materials such as a hydrogel. The IPC solutions were then left to gel for 15 min before being blotted for 4 s and then plunge frozen using Gatan CP3 in liquid ethane (prepared by cooling the gas in liquid nitrogen). The grids were assessed using a CLEM stage (CMS.
196, Linkam, UK) using Nikon upright Light microscope (Nikon, Japan). Grids with areas thin enough to be analysed were transferred to Gatan cryo-stage using a Gatan 915 cryo-holder. The samples were imaged in JEM 2100 Plus TEM (JEOL, USA. Images were analysed using Gatan US1000XP CCD camera and Gatan GMS 3.

3.2.3.7.3 Cryogenic Focused Ion Beam Scanning Electron Microscopy (Cryo-FIB-SEM)

IPC (~40 mg/mL collagen) solution (10 μL) was loaded into a brass ring (3 μm internal diameter) and DI water (30 μL, prewarmed at 37°C) was pipetted on top, followed by a 30 min incubation at 37°C. Any remaining water was removed, and the rig was then slam frozen in liquid nitrogen using a metal-mirror cryofixation unit (Leica Reichert Jung MM80E, Germany). The brass ring was transferred into a cryo-SEM preparation chamber (Quorum PP3010, UK). The whole Cryo-FIB-SEM preparation and imaging process is simplified in a flow diagram in Figure 41.

The sample was then first coated with platinum (for 6 s at 10 mA) and then moved into the cryo-stage where it was then coated with a 3 μm thick layer of organometal platinum (CpPt(CH)3). Three rectangular areas (10x30 μm dimension) of the coated sample where subsequently selected based on being frost free. The milling of the locations was conducted using a gallium based focused ion beam at a high voltage. The sample was transferred back to the Cryo-SEM preparation chamber and the temperature was increased -90°C to allow for sublimation of water occur over 30 min. The sample moved was back to the cryo-stage and images were then taken using a Quanta 200 3D SEM (10 kV, Quanta, US).
3.2.3.8 Statistical Analysis

The data sampling was carried out and statistically analysed as described in section 2.2.3.5. The samples in this chapter were prepared in triplicates and the experiments were repeated on at least 3 separate occasions. Also, the error bars represent the SE as the n number was at least n=3. ORM-ANOVA was used to analyse the hydroxyproline and Pierce BCA assay data. F-ANOVA was utilised to analyse the remaining data. A minimum significance level of p< 0.05 was used for all statistical tests. 

**Figure 41.** A flow diagram describing the sample preparation and imaging process in Cryo-FIB-SEM.
3.3 Results

3.3.1 Preparation and Optimisation of IPC

The choice of collagen for the preparation of IPC was pepsin soluble (PS) bovine corium type I collagen due to its superior biocompatibility compared to AS collagen \[29\]. A method for preparing an IPC solution using acid soluble (AS) bovine corium collagen type was also investigated to find out whether the two preparations behave differently. EDTA stabilised the IPC at pH 7 and allowed the collagen to remain in solution.

The IPC preparation process involved precipitating the collagen from NaCl (0.8 M) as collagen is insoluble at that concentration of NaCl (Figure 42A). After dialysis in acetic acid (0.1 M), dialysis was followed with a series of EDTA solutions at increasing pH levels (pH 5-7) to form a clear IPC solution (Figure 42B). The incremental increase in pH allowed EDTA to bind to collagen (Personal Communication with Dr Dale P Devore). The collagen appeared to be soluble and stable at pH 7.

![Figure 42. The appearance of IPC mixture during preparation. A) Salt precipitated collagen before dialysis and B) Clear IPC solution after the dialysis is completed.](image)

The dialysis bag was deflated compared to the start of the dialysis process (Figure 42B). Dialysis bag deflation was due to the inclusion of 3.5% mannitol and 100 mM sucrose which results in the movement of water from inside the dialysis bag to compensate for a change in the osmotic potential. The osmolarity of the IPC solution was found to be approximately 449.1 (±55) mOSm/kg. This value fell within the well tolerated range (200-600 mOSm/kg) for intramuscular and subcutaneous formulations \[200\].
The IPC appeared as a clear, viscous solution due to the high concentration of collagen (15-50 mg/mL). A positive displacement pipette was used to handle the IPC solution. To trigger fibrillogenesis the IPC solution can be added to a vial followed by the addition of a biologically compliant media such PBS at a 1:2 ratio (IPC: PBS) or higher. The solution changed from a clear viscous solution to white solid gel (Figure 43). The gel opacity increased with time which indicates the growth of the collagen fibrils as the EDTA diffuses out. A thin layer of fibrils forms formed almost immediately on the outside of the gel and the kept growing towards the centre with time as more EDTA diffuses out of the IPC \[194\]. It was shown that when an IPC solution was incubated at 37°C without any dilution, fibril formation (the solution turned cloudy) was triggered. This was also reversible when the temperature was dropped before further gelling could occur. This indicated the stabilisation process is temperature dependent and fibrillation might be somewhat reversible in the early stages.

![Figure 43. The changes in the appearance of IPC with gelling time. A) Minor changes in IPC over during the 15 min gelling time. B) IPC solution in PBS before and after fibril formation was completed.](image)

### 3.3.1.1 Effect of Excipients

Buffers such as PBS are widely used with proteins to maintain pH. Sodium azide at very low concentration (15 mM) is often used in preclinical drug delivery studies to prevent microbial growth. Since the aim of preparing the IPC was to formulate therapeutics, the effects of these excipients on the IPC was evaluated. At a concentration of 15 mM, sodium azide did not affect the
gel stability and the gel seemed to remain visually stable after 30 days of storage at 4-8°C. The gelling time of the IPC solution mixtures did not appear to visually change when compared to an IPC solution without the PBS or sodium azide.

A 20 kDa PEG was also incorporated in the IPC solution to investigate whether its addition would affect the stability and gelling properties of the collagen in an IPC. PEG associates with large number of water molecules which would potentially allow for use of less collagen while maintaining suitable mechanical properties for fibrillogenesis and depot formation. A 20 kDa PEG would potentially be large enough to mix with the collagen molecules and affect the gel mechanical properties. A solution of PEG was added at different ratios and polymer concentrations. PEG could be mixed with collagen to a final PEG concentration of a maximum of 2.5 mg/mL. Above that concentration, the IPC started forming collagen fibrils immediately and formed a gel inside the syringe during the mixing process. The mixing could be conducted at different ratios of IPC to PEG solution varying from 1:10 to 1:4. The concentrations were varied but the outcomes all showed that final concentration of 2.5 mg/mL of PEG in IPC was the best concentration at maintaining the IPC gelling properties. The IPC solution viscosity visibly increased after the mixing process. The mixture remained clear and formed a gel after 30 days of storage at 4-8°C.

### 3.3.1.2 Effects of IPC Dilution and Reduction in EDTA Concentration

For an IPC solution to be formulated with either drugs or cells, the formulation process would involve dilution of the collagen concentration. The extent to which dilution might be possible and the effect on the gelling properties of the IPC was analysed. Solutions in 35 mM EDTA could be diluted with a solution of 15-35 mM EDTA (in 100 mM sucrose and 3.5% D-mannitol) at up to 1:4 of gel to liquid. When the ratio exceeded 1:4, the IPC collapsed and formed fibrils immediately inside the syringe during mixing. Blobs of insoluble opaque aggregates formed. This observation was the same regardless to whether the EDTA solution contained other additives like PBS or PEG. These different solutions formed gels with the gelling time increasing with dilution (decrease in collagen concentration).
The possibility of producing an IPC using less EDTA was also explored as EDTA can affect cell behaviour and enzyme function \[201\]. It was observed that the IPC from PS bovine corium collagen can be prepared at 15 mM EDTA concentration and it remained stable after 24 h at ambient temperature. However, the IPC prepared from AS bovine corium collagen was only stable at a minimum of 20 mM EDTA (in 100 mM sucrose and 3.5% D-mannitol, at pH 7.2) and remained in solution even after 24 h at an ambient temperature.

### 3.3.2 Collagen Content

Hydroxyproline and BCA assays were used to determine the collagen concentration in IPC. The pH of the solution (Figure 44, next page) did not have a significant effect (p = 0.07) on the albumin concentration calibration curve. The PS bovine corium collagen standard curve was significantly different (p < 0.001) to those of albumin at both pH values. The collagen in the IPC gel was solubilised at pH 11 which meant that effect of pH on the albumin standard curve need to be analysed. The absorbance readings of the collagen standard curve were lower at all protein concentrations.

A commonly used assay which measures collagen concentration is based on the content of hydroxyproline in the sample. Hydroxyproline is unique to collagen and its proportion in collagen is conserved from collagens from different sources (12-14%) making the hydroxyproline assay a highly sensitive assay for collagen \[202\]. Its cost is an issue and the development of an alternative cheaper assay would therefore be ideal. The BCA assay is based on use of Cu^{2+} ions which become reduced to Cu^{+} ions by the amide bonds in proteins like collagen. The colour changes from green to purple blue. The intensity of the blue colour is an indication of the protein concentration \[203\]. The BCA assay was investigated for use as an alternative to the hydroxyproline assay to determine the collagen concentration.
The hydroxyproline assay underestimated the collagen concentration (Table 13, next page) (p< 0.001) in those IPC batches. The hydroxyproline assay indicated that the collagen concentration in the different IPC batches varied from 24-50 mg/mL (Table 13, next page). The BCA assay did not show any pattern or resemblance to the values obtained using the hydroxyproline assay. The extracted values of collagen concentration (14-34 mg/mL) reported using the BCA assay showed a high variability and were all much lower than those calculated from the hydroxyproline assay. The B1-PS batch contained 30.93 (± 5.39) mg/ml collagen according to the BCA assay and 50.1 (±0.09) mg/mL according to the hydroxyproline assay.

Figure 44. Pierce BCA assay standard curve of collagen and albumin. pH 11 albumin: y = 0.001x + 0.215, $R^2 = 0.987$, albumin pH 7: y = 0.001x + 0.207, $R^2 = 0.982$ Collagen: y = 0.0005x + 0.141 $R^2 = 0.994$. The values represent the mean ±SE and at n=3. p< 0.001 when the albumin curves were compared to the collagen curve using F-ANOVA. * = Statistically significant (p < 0.05).
Table 13. The difference in collagen concentrations IPC reported by using the BCA and hydroxyproline assays. B= batch number, PS= pepsin soluble and AS= acid soluble collagen. Values represent the mean ±SE and at n=3.

<table>
<thead>
<tr>
<th>IPC batch</th>
<th>Collagen concentration (mg/mL)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pierce BCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1-PS</td>
<td>30.93 (± 5.39)</td>
<td>50.1 (±0.09)</td>
<td></td>
</tr>
<tr>
<td>B2-PS</td>
<td>23.61 (±3.58)</td>
<td>25.99 (±1.59)</td>
<td></td>
</tr>
<tr>
<td>B3-PS</td>
<td>15.52 (±3.8)</td>
<td>37.24 (±0.15)</td>
<td></td>
</tr>
<tr>
<td>B4-PS</td>
<td>20.24 (±2.75)</td>
<td>48.65 (±0.41)</td>
<td></td>
</tr>
<tr>
<td>B5-PS</td>
<td>34.07 (±2.62)</td>
<td>43.93 (±0.11)</td>
<td></td>
</tr>
<tr>
<td>B1-AS</td>
<td>14.25 (±3.00)</td>
<td>23.82 (±0.45)</td>
<td></td>
</tr>
</tbody>
</table>

3.3.3 In-vitro Collagen Degradation

The in-vitro degradation of fibrillated PS bovine corium collagen in IPC vs that from a fibrillated PS bovine corium collagen (neutralised) is shown in Figure 45. The degradation of collagen by the collagenase enzyme was much faster in PS collagen gelled through the standard method of neutralisation and was significantly different (p< 0.001) to that of collagen gelled prepared by the IPC method. At 1h, 13.6 % (±0.3) of the naturally gelled PS collagen was degraded while only 2.2% (±2.2) of the collagen IPC gel was observed to have degraded. After 24 h the PS bovine corium collagen gel was fully degraded. Only ~8.0% of collagen in the IPC gel had degraded. As some EDTA remains in the IPC, resistance collagenase degradation may have occurred.
3.3.4 Mechanical Characterisation of IPC

The mechanical properties of the IPC gel were analysed (Figure 46) to further characterise the IPC. It was shown that the viscous modulus (G'', loss modulus) was significantly higher (P< 0.001) than the elastic modulus (G', storage modulus). The IPC gel was a material which was more viscous than elastic in its mechanical property. The mechanical stiffness (G*, complex modulus) of the gel was higher than both viscous and elastic modulus. The average complex modulus increased from around 1570 Pa (±79) at 0.1 Hz to 1700 Pa (±76) at 10 Hz. The viscous modulus, on the increased from 800 (±27) to 1400 Pa (±47). The elastic modulus remained around 700 Pa.

**Figure 45.** The in-vitro degradation profile of collagen in neutralised PS (pepsin soluble) collagen gel compared to the IPC (in-situ polymerisable collagen) gel at 37°C. Degradation levels were measured using the hydroxyproline assay. The values represent the mean ±SE and at n=3. *p< 0.001 when the two profiles were compared using F-ANOVA. * = Statistically significant (p < 0.05).
3.3.4. Effect of Syringe Mixing

The aim of the IPC solution preparation was to subsequently formulate it with therapeutics. The high IPC solution viscosity means that a mixing method was required to be developed. It was found that IPC solutions mixed using two syringes did not have any effect on the mechanical properties of the gel. The syringe mixing process did not reduce the mechanical stiffness (complex modulus) of the gel ($p=0.872$) when IPC was mixed using the syringe mixing method for 2 and 6 min (Figure 47). The mixing time (2 and 6 min) did not have a significant effect ($p=0.43$) on the mechanical strength. All gels increased in mechanical strength ($G^*$) with an increase in the oscillation frequency ($p<0.001$). As a result, the syringe mixing method can be used to formulate IPC with therapeutics without affecting the gel mechanical properties.

**Figure 46.** The mechanical properties profile of the IPC gel. $G'$= elastic modulus, $G''$= viscous modulus, $G^*$= complex modulus. Measurements were conducted at 37°C. The values represent the mean ±SE and at $n=3$, $p<0.001$ when the profiles were compared using F-ANOVA. * = Statistically significant ($p < 0.05$).
The effect of collagen concentration on the complex modulus (mechanical stiffness) of the IPC gel was also investigated. The collagen concentration had a significant effect ($p < 0.001$) on the IPC gel mechanical stiffness (Figure 49). The mechanical stiffness of the IPC gel more than doubled in value when the collagen concentration was increased from 26 mg/mL to 37 mg/mL. The complex modulus of increased at a similar rate for both gels with the increase in oscillation frequency ($p < 0.001$). The effect of the collagen concentration on the elastic and viscous moduli is shown in Figure 50. There was a statistically significant difference between the viscous modulus ($p< 0.001$) and the elastic modulus ($p< 0.001$) at the different concentrations. The viscous modulus was almost two times higher than the elastic modulus for both collagen concentrations. A reduction in collagen concentration resulting in lower fibril density resulting in lower mechanical stiffness.

**Figure 47.** The effect of syringe mixing times on the gel mechanical stiffness (complex modulus) of IPC. Measurements were conducted at 37°C. The values represent the mean ± SE and at $n=3$, $p< 0.001$ when the profiles of the mixed gel and the un-mixed gel were analysed using F-ANOVA. * = Statistically significant ($p < 0.05$).

### 3.3.4.2 Effect of Collagen Concentration

The effect of collagen concentration on the complex modulus (mechanical stiffness) of the IPC gel was also investigated. The collagen concentration had a significant effect ($p < 0.001$) on the IPC gel mechanical stiffness (Figure 49). The mechanical stiffness of the IPC gel more than doubled in value when the collagen concentration was increased from 26 mg/mL to 37 mg/mL. The complex modulus of increased at a similar rate for both gels with the increase in oscillation frequency ($p < 0.001$). The effect of the collagen concentration on the elastic and viscous moduli is shown in Figure 50. There was a statistically significant difference between the viscous modulus ($p< 0.001$) and the elastic modulus ($p< 0.001$) at the different concentrations. The viscous modulus was almost two times higher than the elastic modulus for both collagen concentrations. A reduction in collagen concentration resulting in lower fibril density resulting in lower mechanical stiffness.
**Figure 48.** The change in mechanical stiffness (complex modulus) with increase in collagen concentration in the IPC gel. The IPC collagen concentration was 26 and 37 mg/mL and measurements were conducted at 37°C. The values represent the mean ±SE and at n=3. p< 0.001 when the different concentration profiles were compared using F-ANOVA. * = Statistically significant (p < 0.05).

**Figure 49.** The modification in the viscous and elastic modulus profile of the IPC gel due to the change in collagen concentration (26 to 37 mg/mL). G’ = Elastic Modulus and G’” = Viscous Modulus. The measurements were conducted at 37°C. The values represent the mean ±SE, n=3. p< 0.001 when the different concentration profiles were compared using F-ANOVA. * = Statistically significant (p < 0.05).
3.3.4.3 Effect of IPC Dilution

The aim was to formulate the IPC solution in subsequent stages, and it was previously determined that a 1:10 (solution: IPC) mixing ratio was required to achieve a homogenous mixture. The effect of diluting an IPC solution by 10% (in 35 mM EDTA solution, pH7.2) on the gel mechanical properties was analysed as a result. The 10% dilution in concentration resulted in almost a 20% reduction (Figure 50) in the mechanical stiffness of the IPC gel (p< 0.001). The mechanical stiffness profiles increased to a similar extent with the rise in the oscillation frequency (p< 0.001). When formulating IPC with other solutions at 1:10 ratio this decrease in mechanical stiffness needs to be considered.

![Complex Modulus vs Frequency](image)

**Figure 50.** The change in mechanical stiffness (complex modulus) of the IPC gel due to a 10% dilution in collagen concentration. Measurements were conducted at 37°C. The values represent the mean ±SE and at n=3. p< 0.001 when the two profiles were compared using F-ANOVA. * = Statistically significant (p < 0.05).

3.3.4.4 Effect of Lyophilisation

A freeze-drying method was also established to lyophilise the IPC as it will improve storage stability. An aqueous solution means that drug stability might be compromised. Freeze drying helps to avoid the need to store the formulation in a pharmaceutically acceptable non-aqueous liquid. A freeze-
dried drug formulation means the effect on drug degradation and properties are lower when storage is in an aqueous environment. Stability was the main reason for lyophilisation of the IPC. If the IPC can be freeze-dried it can further improve the product application due to less stringent storage and shipping requirements. [204].

The possibility of freeze drying the IPC solution was evaluated (Figure 51) and the properties of the IPC did not seem to change. The IPC could be reconstituted within 1 min (pipetted up and down 10×), and the gel remained clear (Figure 51B) and stable after reconstitution. The reconstituted gel remained stable (clear) and could form a gel, even after 1 month of storage at 4°C. The reconstituted solution could also be gelled successfully (Figure 51C) using the same length time as an IPC that is not lyophilised.

Due to an increased surface area, it was observed that when the lyophilised pellet was cut into small pieces the reconstitution step occurred in half the time taken than when the pellet is uncut (pipetted up and down 5× for <1 min). Also, a decrease in collagen content resulted in the pellet being reconstituted faster than a pellet with a higher collagen content.

**Figure 51.** The reconstitution and gelling of lyophilised IPC. **A)** before reconstitution, **B)** after reconstitution with DI water and **C)** after gelling of IPC with DI water.
The effect of lyophilisation on the mechanical properties of the IPC gel was also evaluated (Figure 52) and the complex modulus remained relatively unchanged and there was no statistical difference between the two profiles (p=0.83). The mechanical stiffness increased with the rise in oscillation frequency (p<0.001) in a similar manner for both samples.

![Graph showing complex modulus vs. frequency before and after lyophilisation](image)

**Figure 52.** The effect of lyophilisation on the mechanical stiffness (complex modulus) profile of the IPC gel. Measurements were conducted at 37°C. The values represent the mean ±SE and at n=3. p=0.83 when the two condition profiles were compared using F-ANOVA. N/S = Not statistically significant (p > 0.05).

### 3.3.5 Morphological Characterisation

Imaging allows the ability to better visualise the morphology and the 3D structure of hydrogels which can be useful for depot and formulation design. Scanning electron microscopy (SEM) is often used to image hydrogels, however, the sample must be under vacuum to be analysed. The samples must first be thoroughly dried which is an issue as both direct air-drying and freeze-drying can result in considerable distortion of the specimen morphology and information about the hydrated state is lost. The high surface tension of water (727.5 µN/cm² at 20°C) upon evaporation results in high levels of drying artefacts in the sample which result in an inaccurate representation of the gel morphology and internal structure. Ice-crystal formation during the
lyophilisation process also distorts the sample making it difficult to analyse accurately \[205\].

Cryogenic Focused Ion Beam SEM (Cryo-FIB-SEM) can potentially be a good alternative to obtain a more detailed image of the structural morphology of the IPC gel in its native hydrated state. The process first involves rapidly sample freezing under pressure to prevent the water from freezing (crystallising) to preserve the sample structure in the hydrated, native state. FIB-SEM uses a heavy ion (usually gallium) beam to remove thin (20 nm) slices off the sample surface (i.e. milling). The water in the sample is then sublimed by increasing the temperature from \(-165\) to \(-90^\circ C\). The electron beam in SEM is used to image the sample. If the process is repeated several times a 3D image of the entire sample structure can be produced. Cryo-FIB-SEM can produce 3D image stacks of a volume of thousands of micrometers cubed at an image pixel size down to 5 nm, while still embedded in a hydrated state \[206\]-[208]. The aim of the study was to establish a method to analyse the IPC gel morphology and a cross-section of its 3D structure using Cry-FIB-SEM. Cryogenic transmission scanning microscopy (Cryo-TEM) which combines cryogenic sample preservation and TEM imaging \[209\] was also used as a potential method to monitor the fibrillogenesis of collagen.

### 3.3.5.1 SEM Imaging of Lyophilised IPC

SEM microscopy was carried out for comparison purposes with Cryo-FIB-SEM. The pore size of the gel could not be observed. The only morphology that can observed was the presence or lack of fibrils (Figure 53). The lyophilised IPC solution appeared paper like before fibrillogenesis was triggered (Figure 53A). The fibrils were apparent in the lyophilised IPC gel (Figure 53B).
3.3.5.2 Cryo-TEM Analysis of IPC

Light microscopy was used to choose the IPC gel (mounted on a Holey carbon grid) with an optimal layer thickness ($\leq 2$ µm). A sample layer thickness of less than 2 µm can be imaged by TEM as the electron beams can readily pass through it. In a light microscope the sample appeared transparent (Figure 54) and the carbon grid squares were apparent. These gel areas were thin enough for the electron beam of the TEM to pass through. Areas where the sample was opaque imaging would not be successful. Longer gelling times (> 15 min) produced opaquer and gels with thicker layer (data not shown) and were therefore not analysed with the Cryo-TEM.

**Figure 53.** SEM images of IPC collagen hydrogels A) before and B) after gelling of IPC collagen.
Figure 54. Light microscopy of the difference in thickness of the IPC sample on the carbon grid. Different areas are shown, the opaque cannot be imaged by the Cryo-TEM. Squares= holey carbon grid.

Cryo-TEM showed that the fibril alignment in the PS collagen in the IPC was random (Figure 55A & D). Certain areas of the gel were too thick for the electron beam to pass through, as indicated by the dark areas (Figure 55A). The fibril formation process was observed by the branching of the fibrils (Figure 55B & C) and the changes in the fibril diameter. Early stage fibrillogenesis was also observed as well as ice crystal contaminates. The sample layer thickness of 2 µm limits meant that no more details about the gel morphology could be studied.
The use of Cryo-TEM allowed the monitoring of the fibril formation and the change in fibril diameter (Figure 56). The observed fibril diameter varied from approximately 20 nm to about 200 nm at 15 min gelling time. Additionally, the growth of the fibrils is also shown by the branching process. The regular 67 nm D-banding pattern unique to fibrillar collagen was not observed.
3.3.5.3 Cryo-FIB-SEM analysis

The organometallo-platinum protected the sample and allowed specific areas to be milled (Figure 57A, B & C) without damaging the whole sample with a high voltage beam. In the images the gel layer was grey, and the platinum layer was white (Figure 57D). Three more areas (3 repeats) on the gel were milled in a similar manner to then be taken to the next stage of analysis.

**Figure 56.** The fibril formation process and the changes in collagen fibril diameter. A-D fibrils with different diameters and morphology. Dark grey circular shapes = carbon grid lines. Fibril diameter was analysed by ImageJ.
After the milling process of three rectangular areas (10 x 30 µm dimension) in the sample, the water in the sample was allowed to sublime (at -90°C for 30 min) (Figure 58B). After water sublimation the remaining structures was the actual collagen matrix structure of the gel in an aqueous environment as the low temperature environment (-165°C) allows the imaging of the collagen fibrils inside the IPC without the gel collapsing. The Cryo-FIB-SEM gave a picture of gel morphology in its natural hydrated environment. The images indicate the microscopic collagen fibrils (~40 mg/mL) in the IPC gel are aligned and produce a matrix like system with pore/ mesh size of 300 nm and less (measured by ImageJ software). Due to the limitation in the SEM resolution capacity, the structures could not be resolved any further.

**Figure 57.** The milling stages of the sample in Cryo-FIB-SEM. Gallium ions vaporise a chosen 10x 30 µm area of the sample under high voltage. A-D show the stages of the milling process from the start (A) to the end stage (D).
3.3.6 Summary of results

A reproducible IPC preparation method was successfully developed. A collagen hydrogel with tuneable mechanical properties was also prepared. The syringe mixing method and a 10% dilution can be used in the formulation of an IPC without a significant effect on the gelling properties. Diluting an IPC beyond a 1:4 ratio destabilised the gel. Lyophilisation did not affect the IPC mechanical or gelling properties. Cryo-TEM could be used to monitor fibrillogenesis and Cryo-FIBS-SEM characterised the morphology of the IPC hydrogel.

Figure 58. The gelled IPC morphological structure imaged with Cryo-FIB-SEM. Images of the gel show before (A) and after water sublimation (B, C and D) of water.
3.4 Discussion

The preparation process of the IPC required the optimisation of the volume NaCl solution to collagen ratio to maximise the amount of collagen extracted. The IPC patent did not describe the details of several aspects of preparation process \(^{[105]}\) which meant several optimisation steps had to be carried out to establish a method. The final concentration of collagen in IPC was controlled by varying the amount of water removed of the precipitated collagen after the centrifugation process.

The mechanism by which EDTA stabilises collagen has not been well explored. It is predicted that the negatively charged EDTA molecules sit within the collagen molecules and potentially interact with the positively charged amino acids in the collagen structure resulting in the collagen stabilisation at pH 7. Naika and Tiku have described that the EDTA properties allow it to partially open the protein molecule to form an intermediate state, which can be implicated in protein substrate interactions. This indicates that at pH 7 EDTA might be keeping the collagen molecules apart, therefore in solution, by forming this intermediate stage \(^{[107]}\). Upon exposure to a biologically relevant media like PBS, EDTA can molecules diffuse out allowing fibrils to form (gelling).

The study also showed that a minimum of 15 mM of EDTA is required to stabilise the fibrils in atelocollagen (PS collagen). A 20 mM was required to stabilise the telocollagen (AS collagen) which can be due to the presence of the telopeptides in collagen which result in a higher concentration of EDTA required to form the intermediate stage. IPC solutions with all EDTA concentrations (15-35 mM) were stable for over 24 h when stored at 4-8°C. All IPC batches containing 25-35 mM EDTA were stable for over 24h at an ambient temperature. For EDTA concentrations below 25 mM, the ambient temperature storage stability varied from 6h to over 24h depending on the collagen batch. This can be related to the fact that even though collagen extraction protocols aim to remove polymeric collagen from the final products it is not completely successful. It was shown that the polymeric content of the collagen, age and sex of the animal can vary from batch to batch\(^{[210]}\). These differences have been shown to affect the collagen behaviour. At lower EDTA
concentrations these differences might have an effect on the IPC long term stability at an ambient temperature [210].

Pierce BCA assay was investigated for use to measure the collagen concentration in collagen. The presence of EDTA (a chelating agent) interferes with the method as it can chelate Cu\(^{2+}\) ions which are utilised by the assay [203]. The IPC sample was therefore diluted 50x before analysis. The pH 11 was used to denature the collagen in IPC and expose the amide bonds in the structure. Also, BCA assay works well under alkali conditions, as confirmed by the albumin standard curve (Figure 44). The assay did not accurately predict the collagen concentration (Table 13). This can be due to the EDTA not being diluted enough and the denaturing process of collagen was not complete preventing complete exposure of the amide bonds to the Cu\(^{2+}\). The latter issue might be overcome by first degrading the collagen using a collagenase enzyme which would expose all the amide bonds and followed by carrying out the Pierce BCA assay [211].

The in-vitro degradation of the collagen in IPC by the collagenase enzyme was possible (Figure 45) but occurred at a much slower rate than the collagen gel which had been gelled using the standard neutralisation process. This is mostly due to fact that some EDTA remains inside the collagen structure after the gelling process is completed. It has been shown that around 10 mM of EDTA remains in the collagen structure even after fibrillogenesis is completed [194]. EDTA slows down the degradation process by inhibiting the collagenase enzyme as it chelates the calcium ions which are essential for the enzyme function [212].

To formulate IPC with other mixtures (e.g. drug solutions) mixing the components had to occur. Due to the high viscosity of the IPC, the syringe mixing method was required. The mixing process did not affect the IPC gelling or mechanical properties (Figure 47) due to the non-destructive nature of the mixing method that was developed.

The crosslinking density (or fibril density) of the collagen in IPC gel was increased and therefore mechanical stiffness by increasing the collagen concentration (Figure 49). It is known that increasing the fibril density and hence mechanical stiffness occurs by increasing the collagen concentration. Karimizade, Takallu, and Mirzaei showed that by increasing the collagen
concentration through plastic compression the mechanical stiffness of collagen hydrogels could improve by several orders of magnitude as the fibres become more densely packed. Plastic compression is a method which reduced the water content of collagen gels by the use of compression and a semipermeable membrane [213]. The mesh size and the mechanical properties can also be potentially tuned by the incorporation of biocompatible polymers such as PEG or HA which occupy space in the gel structure and can absorb the extra unbound water.

Recently, Sin Mun et al prepared an injectable hydrogel with tunable mechanical properties based on collagen-chitosan conjugates. The process involved crosslinking collagen type I to chitosan (modified with a phenolic group) by the amine coupling reaction followed by the addition of HRP enzyme and H₂O₂. The mechanical properties were tuned by varying the collagen to chitosan ratio. Although promising, the fact that the gelling process starts immediately after the addition of HRP enzyme and H₂O₂ before injection which might cause issues. For example, it can limit how much the mechanical strength can be increased before the product is no longer injectable [214].

Surprisingly the IPC gelling (Figure 51) and mechanical (Figure 52) properties were not affected by lyophilisation. As well as stabilising the IPC solution sucrose acts as lyoprotectant and mannitol acts as a bulking agent when used in lyophilisation. Both molecules allow for a successful freeze-drying which results in a rugged cake that does not collapse during primary drying, and the existence of an amorphous phase where molecules of excipient are free to interact with the protein [215].

Imaging techniques (Cryo-TEM and Cryo-FIB-SEM) were utilised to provide a rational design and characterisation of collagen-based biomaterials are essential to further investigate as they allow a better tuning of the biomaterial properties early in the development stages. Cryo-TEM was ideal for monitoring fibrillogenesis of the collagen in IPC (Figure 55 and Figure 56). It allowed direct investigation of the IPC at a certain stage of fibrillogenesis in its vitrified, frozen-hydrated state, i.e. very close to the hydrogel native state[209]. The lack of negative staining with a heavy metal like uranyl acetate did not allow the visualisation of or changes to the D-banding pattern in the collagen [216]. Quan and Sone successfully imaged the collagen fibril alignment and the
D-banding patterns in cry-sectioned tissues of rat tail tendons using Cryo-TEM. Cryo-sectioning allowed for 2 μm samples to be produced. Staining with uranyl acetate allowed the visualisation of the collagen banding patterns \cite{217}.

Another technique which can also be used to monitor collagen fibrillogenesis is Second Harmonic Generation (SHG) microscopy. SHG microscopy is a multiphoton imaging technique which relies on the tight alignment of peptide bonds along the collagen triple helix and within fibrils. A method of analysis was established by Bancelin et al which allowed the imaging of the kinetics of the fibrillogenesis process and the continuous monitoring of the process in the same sample \cite{218}.

Cryo-FIB-SEM provided a defining image of the internal morphology of the IPC (Figure 58). Cryo-FIB-SEM allowed for the removal of the vitreous water and the imaging of the gel structure in its native state. The IPC structure was matrix like, and the mesh size was less than 300 nm which is due to the collagen fibril formation. Due the resolution limit in the SEM equipment itself, smaller structures were unresolvable. It indicates that IPC can potentially trap nanoparticles.

It is mathematically possible to estimate the mesh size of hydrated hydrogel based on equilibrium-swelling theory and rubber-elasticity theory. In the case of highly hydrated hydrogels, where the cross-links are introduced in solution (e.g. fibrillogenesis), the mesh size (ξ) can be calculated according to the following equation:

\[
\xi = Q^{1/3} (C_nNL^2)^{1/2}
\]

\(Q\) = volumetric swelling ratio of the hydrogel
\(C_n\) = Flory characteristic ratio of the polymer (or collagen)
\(N\) = number of bonds between two cross-links
\(L\) = length of the bond along the polymer backbone.

\(C_n\) and \(L\) can be calculated from the chemical structure of the polymer chains, but \(Q\) and \(N\) can be experimentally determined by measuring the volume of the hydrogel before and after equilibrium swelling (full gel formation) \cite{219}, \cite{220}.

Critical point drying (CPD) in combination with SEM can potentially be used to image the surface morphology of hydrogels. CPD relies on the
principle that a rise in temperature will cause a liquid held in a sealed chamber to simultaneously expand and evaporate. At the critical point, a certain combination of temperature and pressure, the surface tension becomes zero because the densities of both phases are equal and the boundary between them disappears. The surface morphology of the dried sample can then be analysed using SEM [205]. This technique might not be suitable for IPC gels as it requires organic solvents in which the remnant EDTA is insoluble potentially causes artefacts.
Chapter 4: IPC Formulation to Treat Trichiasis
4. IPC Formulation to Treat Trichiasis

4.1 Introduction

To reduce the risk of blindness, trichiasis treatments vary depending on the degree of severity and include lubrication, eye-lash trephination, laser ablation, and electrolysis or surgery in more severe cases. These interventions have a low long-term success rate and trichiasis recurrence increases with time. There is a real need for therapeutic agents to reduce the scarring process after surgery to prevent blindness \[^{123},^{124}\]. The WHO-GET-2020 SAFE strategy has been established to treat trachoma. It involves promoting facial cleanliness, access to water, mass administration of antibiotics and surgery to treat trichiasis \[^{120}\].

Anti-scarring drugs have been shown to modulate wound healing and reduce scarring \[^{221},^{222}\]. A tissue specific treatment using anti-scarring drugs as a standalone therapy or as adjunct therapy directly after trachoma surgery might provide a significant improvement to the current outcome in trichiasis. An IPC is an excellent candidate to prepare an in-situ gelling slow release depot of anti-scarring agents. It can potentially be injected using a small gauge needle into the subconjunctiva, the palpebral conjunctiva which lines the inside of the eyelids. The drug would be slowly released over time to act locally in the eyelid to prevent scarring. Implant and tissue specific drug delivery can limit systemic drug exposure while maximising the local drug effect. IPC gel might potentially provide an extra support to the eyelid while it slowly degrades over time \[^{90}\].

An anti-scarring drug can in principle be mixed with the IPC as a solution or as a suspension of drug particles. Upon injection the formulation undergoes a solution–gel transition, resulting in a depot which takes the shape of the available space at the injection site causing minimal discomfort \[^{90}\]. The EDTA present in the IPC might potentially have an agonistic or additive effect on trichiasis treatment as it is a known inhibitor of various MMPs \[^{142}\]. An IPC anti-scarring depot could improve treatment outcome as it avoids the frequent dosing that eye drop formulations require. In resource limited parts of the world eye drops are scarce. Moreover, it has been suggested that by coating/entrapping particles inside a hydrogel local inflammation and
phagocytosis of microparticles by macrophages could be prevented \[^{116}\].

Promising anti-scarring drugs (Figure 59) which may be used to formulate a slow release IPC depot are pirfenidone \[^{223}\] and the experimental MMP inhibitors AZ 8955 and ilomastat \[^{133}\]. Several pathways and processes are involved in scarring and wound healing \[^{224},[225]\]. It has been shown that general MMP inhibitors are more ideal as anti-scarring agents than those with more specific drug targets \[^{226}\].

![Figure 59](image)

**Figure 59.** The molecular structure of the anti-scarring drugs: A), ilomastat B) pirfenidone and C) AZ8955.

AZ8955 has a broader MMP inhibitory profile (Table 14) and inhibits more enzymes than ilomastat. Ilomastat is a more effective at inhibiting MMP-1 and MMP-9 than AZ8955. This can be important in the treatment of trichiasis as both enzymes have been found to be elevated in the trichiasis affected tissues of patients \[^{124}\]. Ilomastat and AZ8955 are experimental drugs which have been investigated for a number of conditions \[^{141},[227]\]. Ilomastat a synthetic broad-spectrum MMP inhibitor and is a member of the hydroxamic acid class of reversible MMP inhibitors \[^{228}\]. Ilomastat and AZ 8955 are poorly soluble in water (both 140 µg/mL) \[^{141}\] which have a potent activity against MMP-1, -2, -3 -8 and -9. Its structure contains a modified dipeptide which
resembles a collagen-like backbone to facilitate binding to the active site of the MMP enzyme and a hydroxamate structure (R-CO-NH-OH, where R is an organic residue) which chelates the Zn\textsuperscript{2+} located in the catalytic domain of MMP \cite{227}.

Table 14. A comparison of the MMP inhibition profiles of the AZ8955 compared to ilomastat. MMP selectivity is denoted either by the FRET IC\textsubscript{50} (nM) or the Ki value*. Adapted from Lockwood 2016 \cite{133}.

<table>
<thead>
<tr>
<th>MMP inhibited</th>
<th>Ilomastat</th>
<th>AZ 8955</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>0.4(*)</td>
<td>378</td>
</tr>
<tr>
<td>MMP-2</td>
<td>0.57(*)</td>
<td>0.1</td>
</tr>
<tr>
<td>MMP-3</td>
<td>27(*)</td>
<td>26</td>
</tr>
<tr>
<td>MMP-7</td>
<td>-</td>
<td>966</td>
</tr>
<tr>
<td>MMP-8</td>
<td>0.18(*)</td>
<td>0.8</td>
</tr>
<tr>
<td>MMP-9</td>
<td>0.2(*)</td>
<td>1</td>
</tr>
<tr>
<td>MMP-12</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>MMP-13</td>
<td>-</td>
<td>0.8</td>
</tr>
<tr>
<td>MMP-14</td>
<td>-</td>
<td>28</td>
</tr>
<tr>
<td>MMP-15</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MMP-16</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MMP-19</td>
<td>-</td>
<td>52</td>
</tr>
</tbody>
</table>

Wong et al investigated the effect of subconjunctival injections of ilomastat on bleb survival and the intraocular pressure in rabbits following glaucoma filtration surgery. They found a significant decrease in the visible parameters of scarring by histology of tissue sections in the treated group. The ilomastat-treated tissues maintained a more normal conjunctival morphology. No signs of toxicity or adverse events were observed in ilomastat treated rabbits \cite{229}. Studies have shown the potential of ilomastat in wound healing applications in several animal models without signs of side effects \cite{230}, \cite{231}. Ilomastat also inhibits the growth of Chlamydia trachomatis without inhibiting
the growth of other bacterial cells [232]. Ilomastat can potentially have an anti-microbial effect which can improve to the efficacy of the depot formulation.

Pirfenidone is a pyridone derivative [5-methyl-1-phenyl-2-(1H)-pyridone] and has an empirical formula of C_{12}H_{11}NO. It is an anti-scarring agent which is used clinically in the treatment of idiopathic pulmonary fibrosis (IPF) [233]. The drug inhibits the progression of fibrosis and loss of lung function in patients with IPF via its antifibrotic, anti-inflammatory and antioxidant properties [234]. Pirfenidone is used in combination with nintedanib which a small molecule poorly soluble drug is. Nintedanib works by inhibiting tyrosine kinase, PDGF receptor, fibroblasts growth factor receptor, and VEGF receptor [235].

The anti-inflammatory and ant-fibrotic mechanism of action of pirfenidone is not completely understood. *In-vitro* it inhibits TGF-β-stimulated collagen synthesis, MMP-9, TIMP1 expression [236]. Pirfenidone blocks the mitogenic effect of profibrotic cytokines in adult human lung fibroblasts derived from patients with IPF [233]. Pirfenidone anti-inflammatory properties are related to its regulation of pulmonary inflammatory cytokines and inflammatory cells. Animal models suggest that this occurs by the inhibition of the release of proinflammatory cytokines including IL-1β, IL-6, TNFα and PDGF [237]. The highest daily doses for pirfenidone in IPF is 2.4 g (orally in divided doses) [238]. Although no adverse events were noted in the haematology and blood chemistry profile, common drug related adverse events included nausea, photosensitivity and fatigue [233]. Pirfenidone has a high oral bioavailability and is mainly metabolised by the cytochrome P450 (CYP) enzyme in the liver where around 70–80% of the drug is metabolised by CYP1A2 [237].

Shi et al reported there may also be some therapeutic potential of pirfenidone in cardiac fibrosis *in-vitro*. Pirfenidone reduced the proliferation of cardiac myofibroblasts and attenuated the α-smooth muscle actin expression and collagen contractility in a collagen in an *in-vitro* contraction assay [236]. Recently, pirfenidone was formulated with carboxymethyl pullulan- poly(vinyl alcohol) to prepare slow release microspheres comprised of interpenetrating polymer networks for the treatment of IPF. It was shown to be biocompatible and safe both *in-vitro* and *in-vivo* when given orally to rabbits [239].
4.1.1 Aims

The aim of the work described in this chapter is to design an ocular depot formulation which can release anti-scarring drugs over 14 days. Due to the limited space in the subconjunctiva inside the eyelid the depot volume will maximally be 100 µL. Several formulation approaches will be used to establish a formulation method for an injectable depot. The formulations will be characterised chemically, physically, mechanically and biologically.

4.2 Materials and Methods

4.2.1 Materials

The following materials were purchased from commercial sources. Pepsin soluble type I bovine collagen (Collagen Solutions, UK); rat tail type I collagen (First Link, UK); ilomastat (Stratech Scientific Ltd, UK); AZ5589 (kindly donated by AstraZeneca, UK); pirfenidone (Insight Biotechnology, UK); Live/Dead Cell Imaging Kit (Thermo Fisher Scientific, US); Celltiter-Glo 3D (Promega, US); MMP Activity Assay Kit (ab112147, Abcam, UK); 24-well and 96-well white plate (Thermo Fisher Scientific, US); Poly(lactic-co-glycolic) acid (PLGA) (Purasorb, Netherlands) with a 50:50 weight ratio between the lactic and glycolic monomers (17 kDa); 2,2,2- Trifluoroethanol (Acros Organics, US); human tenon fibroblasts were kindly donated by UCL Institute of Ophthalmology (London, UK).

Penicillin-streptomycin solution (100×) (P/S), Dulbecco's Modified Eagle's medium, Minimum Essential Medium (10×); and foetal bovine serum were purchased from Gibco, UK. Sodium hydroxide (NaOH), phosphate buffered saline tablets, D-mannitol, EDTA disodium salt dihydrate, female luer coupler and dimethyl sulfoxide were purchased from Sigma-Aldrich, UK.

4.2.2 Instrumentation

Bath sonicator (Kerry, UK); Polymax 5 pressure polymerisation unit (Dreve, Germany); EVOS FL light microscope (Thermo Fisher Scientific, US); JASCO LC-2000Plus Series (JASCO, UK); TA Q200 instrument (TA Instruments LLC, USA); Spectrum 100 FT-IR spectrometer (PerkinElmer, USA).
4.2.3 Methods

4.2.3.1 Preparation of IPC Formulations

Different formulation approaches were evaluated for AZ8955 and illomastat, and the most optimal approach was found to be the preparation of a powdered gel formulation. Firstly, a microparticle suspension with different concentrations were prepared by adding a drug powder to an EDTA solution. The solution contains 15-35 mM EDTA, 3.5% mannitol and 100 mM sucrose at pH 7.2. Next, sonication was carried out in a water bath sonicator (Kerry, UK) for 30 min to reduce and homogenise the size of the drug particles. The temperature of the water bath was maintained, and overheating was prevented by the continuous addition of ice. The suspensions were subsequently mixed with the IPC solution at a 1:10 ratio (suspension: IPC) using the syringe mixing method. The final drug concentration was varied between 3 and 6 mg/mL. The air bubbles were removed using the pressure polymerisation at 6.5 bar for 50 min at 21°C.

The pirfenidone formulation was prepared using the same approach, however, the particles completely dissolved at the end of the mixing process to form a clear solution. The highest drug loading of IPC formulations of pirfenidone were 6 and 20 mg/mL (pirfenidone water solubility).

4.2.3.1.1 High Pressure Liquid Chromatography (HPLC) Determination of Drug Content

Standard curves for ilomastat (Appendix Figure 118), pirfenidone (Appendix Figure 119) and AZ 8955 (Appendix Figure 120) were produced by preparing different concentrations of the drugs in PBS. The solutions were then analysed using a HPLC system fitted with a Synergi RP Phenomenex 4-µm, 15-cm C18 column and equipped with an autosampler, a degasser, and two SL bin-pumps. The injection volume was 20 µL.

For ilomastat a flow rate of 1 mL/min was used with acetonitrile and water [containing 0.1% trifluoroacetic acid (TFA)] as eluents A and B respectively. A linear gradient from 80% A to 70% B over 17 min was used. The detection wavelength was 280 nm and the run time was 20 min.
The AZ 8955 HPLC method involved using a flow rate of 1 mL/min with 0.1% TFA in water and methanol as eluents A and C respectively. A linear gradient from 80% A to 80% C over a 20-minute period was used. The detection wavelength was 265 nm and the run time was 25 min.

For pirfenidone a flow rate of 0.7 mL/min was used with water and acetonitrile as eluents A and B respectively. The mobile phase consisted of acetonitrile-water (65:35). The water also contained 0.1% TFA. The UV spectrophotometric determinations were performed at 317 nm with a run time of 10 min.

Drug degradation was investigated by monitoring the appearance of degradation peaks in the HPLC chromatogram. These would appear as additional peak compared to the starting drug peaks.

### 4.2.3.1.2 Drug Loading Determination

AZ 8955 and ilomastat IPC formulation (pre-gelling) were pipetted (5 µL) into 1.5 mL Eppendorf vials. A solution of 0.1 M HCl (10 µL) was mixed with the solution and left for 15 min at ambient temperature. PBS solution (pH 7.4, 235 µL) was mixed the solution which was incubated for a further 15 min. HPLC (section 4.2.3.1.1) was used to determine the drug concentration and the process took in to account the dilution steps.

### 4.2.3.2 In-vitro Drug Release Determination

Drug release was conducted using an in-house made in-vitro ocular release model ([Figure 60](#)) \(^{240}\). The release chamber was connected to an inlet and an outlet. The inlet is connected to an Ismatec peristaltic pump (Cole-Parmer, US) which pumps aqueous media such as PBS (at 37°C) at approximately 2 µL/min. The outlet drains into a 7 mL vial sealed with parafilm to prevent evaporation. The chamber size was 200 µL for all the experimental conditions apart from the 200 µL formulation where a 400 µL chamber was used instead.
Briefly, a 100 µL of each IPC formulation was pipetted into the release chamber, using a positive displacement pipette. PBS solution (100 µL) was then immediately added on top of the IPC formulation to initiate the fibril formation process (gelling). This was done to avoid blockage of the inlet due to the spreading of the IPC formulation before complete gelling occurs due to the slow flow of PBS. The vials are collected at timed intervals and are subsequently analysed for drug content using HPLC. An IPC only control was also loaded into the chamber and the vials were analysed. A control comprised of the particle suspension only was not evaluated as the particles blocked the inlet and outlet ports stopping the flow.

4.2.3.3 Physicochemical Characterisation

4.2.3.3.1 Thermal Analysis

Differential scanning calorimetry (DSC) measurements were conducted to detect phase transitions of samples. Drug free IPC and formulated IPC solutions (containing pirfenidone, ilomastat and AZ 8955) were lyophilised. The samples were weighed (~2mg) into aluminium Tzero pans (T180315, TA Instruments Switzerland) with Tzero hermetic lids (T170404, TA Instruments, Switzerland). An empty pan was used as a reference. The samples were then heated from 20 to 250°C at a rate of 1 °C/min, under a 50 mL/min flow of nitrogen for 28 min. The glass transition temperature (Tg) and melting point (Tm) were analysed using the TRIOS Software v5.0.0 (TA Instruments, US).
4.2.3.3.2 Chemical Composition

Fourier-transform infrared (FTIR) spectroscopy was utilised to investigate the chemical composition of the microparticles, as described in section 2.2.3.4.1.

4.2.3.4 Physical and mechanical Characterisation

4.2.3.4.1 Dynamic Mechanical Analysis

To analyse the mechanical stiffness of the different gel preparations, 350 µL of each formulation was pipetted into 24-well plates using a positive displacement pipetted and then allowed to undergo gelling by the addition of excess PBS. The method of mechanical analysis was as described earlier in section 3.2.3.6.

4.2.3.4.2 Particle Size Analysis

A solution (5 µL) of each IPC formulation was placed on a microscope slide. A cover slip was then placed on top and the samples. The samples were then imaged using light microscope. The particle size was measured using ImageJ analysis software provided in the public domain by the National Institutes of Health [NIH] (MD; http://rsb.info.nih.gov/ij/), based on the micrographs. Approximately 100 particles were selected to determine the mean length of the particle populations. The result was plotted to show the normal size distribution.

4.2.3.5 Preparation of Pirfenidone Encapsulated Microparticle Formulation

4.2.3.5.1 Microparticle Preparation

Pirfenidone microparticles were produced by an electrospraying technique (Figure 61). Previous optimisation steps of the drug polymer solution and the spraying process were carried out to prepare microparticles with a diameter of around 1 µm. Different distances between the nozzle and the collector were used as well as different voltage levels. The composition was also varied by using different concentrations of PLGA and pirfenidone.
The polymer-drug solution composed of 7% PLGA (50:50, 17 kDa) was prepared by dissolving PLGA (360 mg) and pirfenidone (10.58 g) in 2,2,2-trifluoroethanol (TFE). TFE is a volatile solvent commonly used in electrospraying of polymer drug particles [241]. The solution was loaded into a 5 mL syringe and then discharged through a 23-gauge stainless steel nozzle (Nordson, USA). The polymer-drug solution was extruded through a nozzle at a constant rate of 0.3 mL/h using a syringe pump (Cole Parmer, USA). The voltage output was 17 kV, applied between the needle and collector. The tip-to-collector distance was set to 17 cm. The temperature was 22-25ºC and the relative humidity was 23-47%. The charged droplets were discharged from the nozzle in the direction of the magnetic field. As the charged droplets dry up, they are propelled towards the collector which is oppositely charged. The particles which collected on an aluminium foil and were then removed from the surface by a slow scarping using a scalpel. The samples were wrapped in the aluminium foil and stored in a desiccator over phosphorus pentoxide for at least 12 h prior to analysis.

**Figure 61.** The experimental set up for the electrospraying process. 1) The device: A) syringe pump to control the flow rate, B) syringe containing polymer-drug solution, C) aluminium foil collector and D) wire connected to direct current power supply. 2) A simplified diagram of the set-up.
4.2.3.5.2.1 Particle Morphology and Size
To investigate whether spherical particles were formed, the morphology of electrosprayed microparticles was investigated. The particles were mounted on a grid and analysed with SEM as described in section 3.2.3.7.1. The microparticle diameter size distribution was measured by analysing the SEM images with the ImageJ software. Approximately, 100 individual spherical particles were selected to determine the mean particle diameter. The result was plotted to show the normal distribution of diameter.

4.2.3.5.2.2 Thermal Analysis
DSC was used to analyse the phase transitions and thermal properties of the PLGA, pirfenidone and PLGA-pirfenidone microparticles. Powders of the samples were weighed (2 mg) into aluminium Tzero pans and then analysed as described earlier in section 4.2.3.3.1.

4.2.3.5.2.3 Chemical Composition
FTIR was used to analyse the chemical groups and to determine the changes in the chemical composition of PLGA, pirfenidone and PLGA-pirfenidone microparticles. Samples were analysed as described earlier in section 2.2.3.4.1.

4.2.3.5.2.4 Phase Identification
X-Ray Diffraction (XRD) was used to identify the phases differences between PLGA, pirfenidone and PLGA-pirfenidone microparticles. Samples of pirfenidone, PLGA and electro-sprayed microparticles were separately and evenly placed on a slide. Scanning was carried out using a D/Max-BR diffractometer (Rigaku, Tokyo, Japan). The scanning parameters used were a scan speed of $10^\circ$/min at a step of 0.02°. The X-ray diffraction was conducted over the 20 range from 3° to 90°, at 40 kV and at 15 nA.
4.2.3.5.3 Particle Drug Loading and Drug Release from IPC

The particle drug loading was determined by dissolving 10 mg of the PLGA-pirfenidone particles in TFE and the immediately diluting the solution in excess PBS. The mixture was vortexed for 30 s and then the PLGA precipitate was left to settle to the bottom of the vial. Drug content was determined chromatographically as described earlier.

Different amounts of particles were suspended in a 35 mM EDTA solution (in 3.5% mannitol, 100 mM sucrose, at pH 7.2). The particle suspension was then mixed with the IPC solution using the syringe mixing method. The mixing ratio was 1:10 ratio (suspension: IPC) as well. The release study and subsequent analysis was carried out as described earlier.

4.2.3.6 Biological Characterisation

4.2.3.6.1 In-vitro Cytotoxicity of the Drugs and DMSO

The general cell culture protocol for HTF cells was conducted as described in section 2.2.3.3.1. Briefly, a suspension of HTF cells (75 µL, passage 4-9) in complete DMEM were plated into 96-well white plates at a density of 10,000 cells/well overnight (16 h). The complete media contained 1 mM P/S and 10% FBS. Different concentrations of DMSO (1, 5 and 10%) were prepared in complete DMEM. Different concentrations of freshly made drugs (pirfenidone, AZ 8955 and ilomastat) were prepared where the highest concentration of each agent used was according to their water solubility. The highest drug concentration was 140 µg/mL for ilomastat and AZ 8955, and 20 mg/mL for pirfenidone.

Samples of the formulations of AZ 8955 and ilomastat collected on day 7 of the release study were also evaluated for their effect on the cell viability using the same protocol. The released aliquots (in PBS) were filtered using a 0.22 µm Millex-GP syringe filter (Merck, US) and then diluted with complete DMEM media (1:2 drug mixture: media) to a final concentration of 12.5 µg/mL. The drug free control contained the same PBS: media ratio.

After 16 h incubation, the media was removed and drug in DMSO (75 µL) were then added to the wells. The plates were then incubated for 24, 48 and 72 h, in a humidified incubator at 37°C in 5% CO₂. The cell viability was
measured at each time point using the CellTiter-Glo 3D assay, as described earlier in section 2.2.3.3.1.2.

4.2.3.6.2 In-vitro Cytotoxicity of IPC Formulations

HTF cell suspensions (75 µL, 10,000 cells/well and passage 4-7) were plated into a 96 well plate for 16 h where the media was removed at the end of the incubation time. Separately and under sterile conditions, the IPC formulations of AZ 8955 and ilomastat were prepared as described earlier (section 4.2.3.1) with a drug loading dose of 6 mg/mL. Solutions (10 µL) of IPC, IPC-AZ 8955 and IPC-ilomastat were added to the wells. Complete DMEM (290 µL) was added to each well. The plate was incubated for 72 h in a humidified incubator (at 37°C and 5% CO₂) followed by analysis of cell viability as described in section 2.2.3.3.1.2. Controls were cells only and cells incubated with IPC.

4.2.3.6.3 Contraction Assay

Collagen gels were prepared from 80% rat tail collagen (2 mg/mL), 10% MEM (10×) and 10% HTF cell suspension (passage 4-7), as described in section 2.2.3.3.2.1. The cells were seeded in the collagen gels at a density of 6.3 ×10⁴ cells/mL as described by Mohamed-Ahmed et al [141]. The MEM (10×) was added to the collagen solution which was neutralised by adding 1N NaOH. The cell suspension was added to the solution, followed by pipetting (150 µL) into glass bottom MatTek dishes (14 mm well diameter). The collagen solutions were incubated for 10 min at 37°C to allow for the fibrillogenesis process to occur.

Different concentrations of freshly dissolved ilomastat, pirfenidone and AZ 8955H were prepared. The drug solutions were prepared in complete DMEM where the highest drug concentration contained 1% DMSO. The highest drug concentration from each drug was chosen based the cytotoxicity data. Only a concentration which didn’t show any cytotoxicity (cell viability > 90%) was chosen.

To determine the in-vitro bioactivity of the formulations of AZ 8955 and ilomastat collected on day 7 of the release study a contraction assay study was conducted. The samples were filtered and prepared with complete DMEM (1:2)
as above. Controls used included drug free HTF embedded collagen gels, cell free collagen gels and a positive control that was prepared by using complete DMEM which is supplemented with 10% bovine serum albumin (BSA) instead of 10% FBS.

Complete media (for control) or drug solutions (2 mL) were added to the set HTF embedded collagen gels. The gels were detached from the edge of the well and allowed to float using a 10 µL pipette tip. The gels were incubated at 37°C in a humidified incubator with 5% CO₂ for 7 days.

4.2.3.6.4 MMP Activity Assay

The MMP assay was conducted to evaluate the formulations of AZ 89555 and ilomastat released on day 7 from IPC. The total MMP activity was measured by using a FRET-based MMP activity assay, according to the manufacturer's protocol. Briefly, media from the control and drug-treated collagen contraction culture samples (25 µL) were added to a 2 mM p-aminophenylmercuric acetate solution (25 µL). The mixture was incubated at 37°C for 3 h and then the MMP red substrate (50 µL, 1:500) was added. The mixture was incubated for 1 h at an ambient temperature. Fluorescence was measured at excitation/emission of 540/590nm using a plate reader. The study was conducted on day 0, 3, and 7 of the contraction assay study.

4.2.3.6.5 Live-Dead Staining of the Gel

At the end of the contraction assay experiments on day 7, Live/Dead Cell Imaging Kit was used to carry out live-dead staining on the gels. The staining process was according to manufacturer’s protocol and briefly involved adding component A to component B. Component A is a cell-permeable calcein acetoxyethyl ester (Figure 62) and is converted to calcein (by esterase) when it enters live cells. Component B is a cell-impermeable dye which stains dead and dying cells. The stain only enters cells with compromised membranes and produces red fluorescence upon binding to DNA [242], [243]. The reagent mixture was added to the dish at a 1:1 ratio of media to reagent. The MatTek dishes were incubated for 15 min at 37°C in a humidified incubator.
with 5% CO₂. Fluorescence imaging of the gels was conducted using a light microscope. The excitation / emission was 488/515 and 570nm/602 nm.

4.2.3.7 Statistical Analysis

The data sampling was carried out and statistically analysed as described in section 2.2.3.5. In this chapter, the samples were prepared in triplicates and the experiments were repeated on at least 3 separate occasions. Also, the error bars represent the SE as the n number was at least n=3. ORM-ANOVA was used to analyse the data from the cell viability in DMSO and IPC, the contraction efficacy of anti-scarring drugs at specific concentrations and the MPP activity. The remaining data was analysed using F-ANOVA. A minimum significance level of p< 0.05 was used for all statistical tests [160].
4.3 Results

4.3.1 IPC Drug Formulations

Pirfenidone, AZ8955 and ilomastat may be ideal candidates for use as model drugs to prepare a slow release depot formulation that can be used for the treatment of trichiasis. It has been shown that in the subconjunctiva matrix acute remodelling and organisation of scar tissue is observed histologically up to 14 days after subconjunctival surgery. This is unlike the skin where active remodelling and scarring can occur over months. The study described herein then aimed to prepare a formulation with 2-week release profile. The collagen concentration in the IPC used for these studies was ~40 mg/mL which provided a mechanical stiffness of around 1200 Pa at 1 Hz (measured using a plate-plate rheometer). Collagen concentrations higher than 50 mg/mL partially destabilised the IPC and the solution were difficult to mix due to the very high solution viscosity resulting from the increased collagen concentration. Due to high IPC viscosity the syringe mixing method (as described in section 2.2.3.4.1) was utilised to mix the two components. The mixing ratio of the volumes was maintained at a 1:10 ratio (drug mixture: IPC) as it was determined to achieve homogenous mixture without drastically reducing the collagen concentration and therefore mesh size/mechanical stiffness.

The mixing time of the drug mixture and IPC using the syringe mixing method varied depending on the mixture volume and the size of the syringe. To achieve a homogenous mixture of drug particles in IPC solution it was shown that a 3-min mixing time was optimal for a 500 µL mixture using two 1 mL syringes connected by a luer connector. The mixing time was prolonged with the increase in the mixture volume. For example, at 750 µL the mixing time was 5 min. When working at less than 1 mL scale there was a 25% loss in drug during transfer of the drug suspension. The loss in drug occurred when pipetting the drug suspension from the vial into syringes due to the low volume. To achieve a final drug concentration of 6 mg/mL, an 8 mg/mL formulation was used during mixing. The IPC formulation of both AZ 8955 and ilomastat (Figure 63) turned from clear to opaque in the presence of the drug particle suspension. When the IPC solution was mixed with a solution of pirfenidone...
the mixture remained clear (data not shown) because pirfenidone was soluble. The IPC formulation turned white after gelling was initiated (Figure 63C) and the gel remained intact after the release study was completed (Figure 63D). The IPC gel could even be lifted with a spatula without losing its shape and did not visibly change in size.

Figure 63. The IPC appearance before and after powdered gel formulation (in 6mg/mL ilomastat), after gelling and at the end of the drug release process. A) IPC solution before formulation, B) IPC after powdered gel formulation, C) IPC formulation after gelling and D) A fully intact gel inside the drug release chamber.

4.3.2 Physical Characterisation

4.3.2.1 Particle Size

Light microscopy (Figure 64) was used to analyse the morphology of the IPC before and after formulation with pirfenidone, ilomastat and AZ 8955. The drug free and pirfenidone loaded IPC samples appeared clear and particle free in the images. The particles in the ilomastat formulation were rod-like with no sharp edges. The particle in the AZ 8955 formulation were slab-like. The particle size and distribution appeared relatively homogenous. The particle size distributions (Figure 65) were similar for AZ 8955-and ilomastat-IPC formulations both containing 6 mg/mL of drug. The average particle size was 6.65 µm (±1.57) and 6.63 µm (±1.46) for the AZ 8955 and the ilomastat
formulations respectively. The AZ 8955 and ilomastat IPC formulations contained particles which were uniform in size and distribution.

Figure 64. A light microscope image of the IPC solution before and after formulation with anti-scarring drugs at a 6 mg/mL drug loading. A) Drug free IPC, B) Pirfenidone-IPC, C) Illomastat-IPC and D) AZ 8955-IPC powdered gel formulations. 5 μL of each preparation was imaged before gelling was induced.
4.3.2.2 Mechanical Stiffness

Mechanical analysis (Figure 66) was conducted to investigate whether the formulation process or injection with a small gauge needle affects the IPC gel mechanical stiffness. It was shown that the formulation process did not affect the IPC gel mechanical strength. The mechanical stiffness for all preparations increased with the increase in the vibration frequency (p< 0.001, within-subject F-ANOVA). The formulation conditions did not have a significant effect (p= 0.534) on the mechanical stiffness of the gel. The mechanical stiffness of the ilomastat-IPC powdered-gel formulation (6 mg/mL drug loading) was not significantly different (p= 0.516) to the IPC gel. Also, the needle extrusion process did not have a significant effect (p= 0.540) on the mechanical stiffness of the gel formulation.

Figure 65. The particle size distribution of 100 particles in A) ilomastat-IPC and B) AZ 8955-IPC formulations. The average particle size was approximately 7 µm for both formulations (Data extracted from the light microscope images of the IPC formulations).
4.3.3 Physicochemical Characterisation

FTIR and DSC were utilised to analyse the chemical group compositions and the thermal properties receptively of the IPC formulations. The FTIR allows for the identification of drug functional groups in the IPC formulations. FTIR spectra (Figure 67, next page) showed the presence of the functional groups unique to the ilomastat in the IPC formulation indicating successful mixing. The peaks at 3430, 3260 and 2953 cm\(^{-1}\) were due to the N-H, O-H and C-H stretches in ilomastat, and the peaks at 1635 and 1390 cm\(^{-1}\) were due to N-H, and O-H (carboxylic acid) from the IPC component in the formulation. The functional groups unique to AZ 8955 also appeared in the formulation (Figure 68).

**Figure 66.** The effect of formulation conditions on the gel mechanical stiffness. IPC only, Ilomastat-IPC (6 mg/mL drug loading) and the Ilomastat-IPC (6 mg/mL drug loading) extruded using 26G needle size. Measurements were conducted at 37°C. The values represent the mean± SE at and n=3. p= 0.534 when the different profiles were compared using F-ANOVA. N/S= Not statistically significant (p > 0.05).
Figure 67. The FTIR spectra of the chemical group compositions difference in the ilomastat, IPC and IPC-ilomastat samples. The samples were analysed in the solid state at an ambient temperature.
DSC thermograms of both of the ilomastat-IPC (Figure 69) and AZ8955-IPC (Figure 70) formulations indicated that formulations were mixed within the IPC and the drugs each remained in the crystalline state. The IPC showed a degradation peak at 215 °C which matched the thermal degradation point (T_{id}) of collagen in the literature [246]. The IPC thermal denaturation (T_d) was 122°C which was close to the literature value (126°C) of collagen type I in solid state. At this temperature fibrillar collagen denatures in the solid state [247]. The T_d value of the IPC remained the same in the formulation. The melting point (T_m) of ilomastat was indicated by the sharp endothermic peak at 210°C. The ilomastat T_m peak appeared in the formulation but was less pronounced indicating ilomastat also remained crystalline. The T_{id} of collagen was absent in the formulation which can be due to interactions with the ilomastat melting peak which is close in proximity.
The T\textsubscript{m} of AZ 8955 was a sharp endothermic peak at 140°C. The T\textsubscript{d} value of the IPC remained the same in the formulation but was less pronounced due to the closed proximity to the drug melting peak. The T\textsubscript{m} of AZ 8955 appeared in the formulation, although less sharp, which indicated that

**Figure 69.** The DSC thermogram showing the thermal transition of IPC, ilomastat and ilomastat-IPC. All samples were analysed in the solid-state. Ilomastat: T\textsubscript{m}=melting point= 210°C and IPC: T\textsubscript{d}= thermal denaturation= 122°C and T\textsubscript{td}= thermal degradation= 215°C.

The T\textsubscript{m} of AZ 8955 was a sharp endothermic peak at 140°C. The T\textsubscript{d} value of the IPC remained the same in the formulation but was less pronounced due to the closed proximity to the drug melting peak. The T\textsubscript{m} of AZ 8955 appeared in the formulation, although less sharp, which indicated that
AZ 8955 remained crystalline. The \( T_{td} \) of collagen appeared in the formulation at the point (215°C).

**Figure 70.** The DSC spectra showing the IPC thermal transition, the melting point of AZ 8955 on its own and in the formulation. All samples were analysed in the solid-state. AZ 8955 \( T_m \)= melting point= 140°C, IPC: \( T_d \)= thermal denaturation=122°C and \( T_{td} \)= thermal degradation= 215°C.
4.3.4 In-vitro Drug Release in Different Conditions

The *in-vitro* drug release of the formulations was conducted in an open flow rig with a 200-μL chamber (*Figure 71*). The rate of liquid flow through the chamber is set to 2 μL/min (120 μL/h) at 37°C. The outflow of aqueous humour in the eye is not homogenous and varies with the time of the day and age, is on average 2.4 ± 0.6 μL/min (mean ± standard deviation, daytime measurements in adults aged 20–83 years). The human ocular aqueous outflow is close to the flow used in the rig model [248] and is a good estimate of release in rabbits [133]. The formulation would therefore be in non-sink conditions *in-vivo* where drug solubility plays a major role in determine the drug release rate [240].

![Diagram of drug release system](Figure 71)

*Figure 71. The set-up of the drug release system. Drug content is determined using chromatography. Adapted from Parkinson et al. 2012 [240].*

To study the drug release kinetics, data obtained from the *in vitro* drug release studies were normally plotted as percentage cumulative drug release versus time. The equation of the trend line (y= kx+m) was used to establish whether the release profile resembles a zero-order kinetics. If the coefficient of correlation (R²) is ≥ 0.9, the release profile is considered to be zero-order where *k* is the zero-order release constant [249]. A zero-order release profile indicates that the drug release was constant [249], [250].

The effect of different formulation parameters on the drug release from the depot implant formulation was investigated. The effect of the implant size was studied by preparing 100 and 200 μL depot formulations containing 6 mg/mL AZ 8955. The effect of depot drug loading was investigated by varying the concentration of ilomastat (3 and 6 mg/mL) and pirfenidone (6 and 20 mg/mL). The IPC mechanical property was investigated by using gels with a mechanical stiffness of 600 and 1200 Pa where both were loaded with 6
mg/mL of ilomastat. Whether lyophilisation of the formulation pre-gelling affects drug release was also investigated. This was done by lyophilising a 3 mg/mL ilomastat-IPC formulation and then reconstituting it in DI water followed by loading into the release model. Simultaneously the drug release profile of a non-lyophilised formulation was determined at the same time.

4.3.4.1 Effect of the IPC

To investigate whether the IPC slows down drug release IPC was formulated with a solution of pirfenidone. The drug release profile of pirfenidone (Figure 72) showed no change compared to the control. The C$_{\text{max}}$ was achieved at 4 h for both experimental groups. On day 1, the concentration of pirfenidone was 170.9 µg/mL and 149.6 µg/mL for pirfenidone solution and pirfenidone-IPC formulation respectively. The concentration of drug decreased with time (p< 0.001, within-subject F-ANOVA). The IPC did not have a significant effect (p= 0.783) on the cumulative drug release or the concentration of drug (p= 0.587) released over the study period. The IPC did not affect the release of pirfenidone (MW= 185 g/mol) [238].

![Figure 72. The in-vitro drug release profile of pirfenidone-IPC formulation compared to the drug only control at 37°C. The drug loading was 6mg/mL in a 100 µL depot. The values represent the mean± SE and at n=3. Cumulative release: p= 0.783 and Drug concentration: p= 0.587, when the profiles were compared using F-ANOVA. N/S= Not statistically significant (p> 0.05).](image)
Ilomastat and AZ 8955 solutions used in the IPC formulations were prepared at 140 µg/mL drug loading. The drug release profiles for these 2 formulations were also completed within in 2 days as the drugs were mixed as solutions with the IPC. So, the IPC does not affect the release from drug solutions.

### 4.3.4.2 Effect of Drug State

The effect of formulating the drugs in a powdered gel formulation (AZ 8955 and ilomastat) with the IPC at a drug loading of 6 mg solids/mL was analysed. The rig model allowed the formulations release under non-sink conditions to mimic the *in-vivo* environment in the subconjunctiva \[^{133}\]. Formulating powdered gel formulations (~7 µm in average size) ([Figure 74](#) and [Figure 75](#)) reduced the drug release significantly (p< 0.001). As both AZ 8955 and ilomastat are poorly soluble they displayed a prolonged release of 2 weeks. The concentration of drug released for both AZ 8955 and ilomastat was relatively constant. \(C_{\text{max}}\) occurred at 4h for the pirfenidone (383.3 µg/mL) and ilomastat (56.4 µg/mL) formulations. The \(C_{\text{max}}\) was 40.5 µg/mL for the AZ 8955 IPC formulation at 24. A burst release is evident at 4 h ([Figure 74](#)) and was 19.7% for pirfenidone-IPC in contrast to 2.5% and 2.4% in ilomastat-IPC and AZ 8955-IPC, respectively. For the powdered gel formulations, the drug did not have a significant effect on the drug concentration (p= 0.246) or the cumulative drug release (p= 0.206).
**Figure 73.** The change in cumulative drug release in-vitro at 37°C over time for pirfenidone-IPC, ilomastat-IPC and AZ 8955-IPC formulations. The drug loading was 6 mg/mL in a 100 µL depot. The values represent the mean± SE and at n=3. p< 0.001 when the cumulative release profiles were compared using F-ANOVA. * = Statistically significant (p< 0.05).

**Figure 74.** The change in drug concentration of drugs released in-vitro at 37°C over time for the ilomastat-IPC, AZ 8955-IPC and pirfenidone-IPC formulations. The drug loading was 6 mg/mL in a 100 µL depot. The values represent the mean± SE and at n=3. p< 0.001 the drug concentration profiles were compared using F-ANOVA. * = Statistically significant (p< 0.05).
4.3.4.3 Effect of Drug Loading

The effect of drug loading on the pirfenidone-IPC formulation (Figure 75) and powdered gel formulation of ilomastat (Figure 76) was compared. The 100 µL depots of the pirfenidone formulation released the drug rapidly even at a drug loading of 20 mg/mL. The burst release was very high (~20.0%) for both drug loadings. The 6 mg/mL pirfenidone containing IPC formulation released the drug in 2 days but the 20 mg/mL formulation completed the drug release at day 3. Pirfenidone has a moderate water solubility (20 mg/mL) [239]. The increase in drug loading had significant effect (p< 0.001) on the drug concentration but not on the cumulative drug release (p= 0.881).

For the ilomastat-IPC formulation (Figure 76) time and drug loading had a significant effect on both the drug concentration and cumulative release (p< 0.001). This was due to the non-sink conditions and the poorly soluble nature of the drug. An increase in the ilomastat loading (3 to 6 mg/mL) resulted in doubling the drug release period. The 100 µL depot of 6 mg/mL ilomastat-IPC formulation showed a 15-day slow release profile, whereas, the 3 mg/mL formulation showed a 7-day release profile. The burst release at 4 h was low (< 3.0%) for both depots. On day 4, the cumulative release was 66.1% and 43.0% for the 3 mg/mL and 6 mg/mL formulations respectively.
Figure 75. The change in the pirfenidone in-vitro release profile from IPC with increase in drug loading (6 and 20 mg/mL) in a 100 µL depot, at 37°C. The values represent the mean± SE and at n=3. Cumulative release: p= 0.881 and drug concentration: p< 0.001 when the data was analysed using F-ANOVA. N/S= Not statistically significant (p > 0.05). * = Statistically significant (p< 0.05).

Figure 76. The change in in-vitro drug release of the ilomastat-IPC formulation with an increase in ilomastat loading in a 100 µL depot, at 37°C. Drug loading was 3 and 6 mg/mL. The values represent the mean± SE and at n=3. p values < 0.001 for both cumulative release and drug concentration profiles when analysing the data using F-ANOVA. * = Statistically significant (p< 0.05).
4.3.4.4 Effect of Implant Size

To analyse the effect of depot size on AZ 8955 drug release (Figure 77), 100 and 200 µL depots were formulated and to investigate whether the depot could be delivered to other parts of the eye or body. The burst release at 4 h was again low (< 3.0%) for both conditions. The cumulative drug release was significantly different (p< 0.001) between the two experimental conditions. Time had a significant effect (p< 0.001, within-subject F-ANOVA) on the drug release profile. The C<sub>max</sub> was achieved at 4h and 24 h for the 200 µL (47.0 µg/mL) and the 100 µL (38.3 µg/mL) implants respectively.

The 200 µL depot AZ 8955-IPC released over 25 days, compared to 15 days of 100 µL depot at 6 mg/mL drug loading. The drug concentration of AZ 8955 released over the study period varied significantly over time for both implant sizes. On day 1 the drug concentration released was 42.6 µg/mL and 38.3 µg/mL for the 100 µL and 200 µL depots respectively. On day 15 the concentration was 4.4 µg/mL and 16.6 µg/mL µg/mL for the 100 and 200 µL respectively.

**Figure 77.** The effect of implant size on the AZ 8955-IPC formulation in-vitro drug release, at 37°C. The drug loading was 6 mg/mL in 100 µL and 200 µL depots. The values represent the mean± SE and at n=3. p values < 0.001 for both cumulative release and drug concentration when the profiles were analysed using F-ANOVA. * = Statistically significant (p< 0.05).
4.3.4.5 Effect of Lyophilisation

The drug release profile (Figure 78) of lyophilised and un-lyophilised IPC formulations was determined as it is envisaged an IPC drug formulation stored in solution would have challenges for maintaining API stability. Ilomastat has been shown to degrade over time when stored in aqueous solutions [133]. The cumulative drug release of a lyophilised formulation was very similar to that of a non-lyophilised formulation (Figure 78). The formulations were composed of 100 µL depots, loaded with 3 mg/mL ilomastat in an IPC gel. The lyophilisation process did not have a significant effect (p= 0.963) on the cumulative drug release or the concentration profile (p= 0.288). Time had a significant effect (p< 0.001, within-subject F-ANOVA) on the drug release profile.

The burst release was again low (< 3%) and the subsequent drug release occurred over 7 days. For instance, the concentration of ilomastat released on day 1 was 15.6 and 28.2 µg/mL for the un-lyophilised and the lyophilised formulations respectively. The cumulative release was 18.7% and 23.3% for the un-lyophilised and the lyophilised formulations respectively.

![Figure 78. The effect of lyophilisation on the in-vitro drug release profile of the ilomastat-IPC formulation compared to the un-lyophilised formulation at 37°C. The drug loading was 3 mg/mL in a 100 µL depot. The values represent the mean± SE and at n=3. Cumulative release: p= 0.963 and Drug concentration: p= 0.288 when the data in the two conditions were analysed using F-ANOVA. N/S= Not statistically significant (p > 0.05).]
4.3.4.6 Effect of IPC Gel Mechanical Stiffness

An increase in collagen concentration strengthens the gel mechanical stiffness due to reduction mesh gel size due to the higher fibril density \[^{251}\]. Diffusion of molecules like O\(_2\) has been shown to be affected by collagen gel concentration \textit{in-vitro} \[^{252}\]. The effect of the IPC gel mechanical stiffness and potentially its pore size on the drug release of ilomastat (Figure 79) was therefore studied.

Doubling of the gel mechanical stiffness did not affect the drug release profile of the ilomastat-IPC formulation. The drug release was from depots containing 6 mg/mL ilomastat in gels with a mechanical stiffness of 600 and 1200 Pa. Time had a significant effect (\(p< 0.001\), within-subject F-ANOVA) on both the drug concentration and cumulative release. In both gels the cumulative drug release occurred slowly over two weeks (15 days). The mechanical stiffness did not have a significant effect on the cumulative drug release (\(p= 0.486\)) or the concentration of drug released (\(p= 0.246\)). The \(C_{\text{max}}\) occurred at around 4h and was around 60 µg/mL for both formulations.

![Graph showing drug concentration and cumulative release](image)

**Figure 79.** The effect of gel mechanical stiffness (complex modulus) on ilomastat \textit{in-vitro} release from the IPC formulation at 37°C. Formulations were prepared from IPC preparations with a gel mechanical stiffness of 600 & 1200 Pa. The drug loading was 6 mg/mL in a 100 µL depot. The values represent the mean± SE and at \(n=3\). Cumulative release: \(p= 0.486\) and drug concentration: \(p= 0.246\) when data from the two conditions were compared using F-ANOVA. N/S= Not statistically significant (\(p > 0.05\)).
4.3.4.7 Summary of Release Kinetics of the Formulations

The release kinetics of the different formulations are summarised in Table 15. The AZ 8955 and the ilomastat formulations approximately follow a zero-order release kinetic (the $R^2 \geq 0.9$). The pirfenidone formulations did not follow a zero-order release kinetics ($R^2 << 0.9$). The $t_{1/2}$ for 1200 Pa and the 600 Pa ilomastat-IPC (6 mg/mL) was 5.4 and 4.9 days respectively. The $t_{3/4}$ was 8.2 and 7.8 days for the former and the latter respectively.

The 3 mg/mL ilomastat-IPC displayed $t_{1/2}$ and $t_{3/4}$ values of 3.1 and 4.5 days respectively which was much shorter than at 6 mg/mL, due to the decrease in drug loading. When the formulation was lyophilised the $t_{1/2}$ and $t_{3/4}$ values were the same as before lyophilisation. A 100 µL of the AZ 8955 -IPC formulation which contained 6 mg/mL of drug had $t_{1/2}$ and $t_{3/4}$ values which were 4.6 and 7.6 days respectively. When the depot volume was increased to 200 µL (6 mg/mL drug loading) the values were 9.4 and 14.9 days respectively.

The average flow rate of the media for the different formulations was 93.8-117.5 µL/h.
Table 15. The Release kinetic profiles for different IPC formulations. All formulations were prepared using an IPC which achieves a 1200 Pa mechanicals stiffness and a 100 µL depot, unless otherwise stated.

<table>
<thead>
<tr>
<th>Formulation (+ drug concentration)</th>
<th>Trend line equation</th>
<th>Intercept</th>
<th>Slope</th>
<th>R²</th>
<th>T&lt;sub&gt;1/2&lt;/sub&gt; (days)</th>
<th>T&lt;sub&gt;3/4&lt;/sub&gt; (days)</th>
<th>x flow rate (mL/h)</th>
<th>Flow rate variation (mL/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ilomastat-IPC (6 mg/mL)</td>
<td>y = 7.14x + 11.58</td>
<td>11.58</td>
<td>7.14</td>
<td>0.936</td>
<td>5.4</td>
<td>8.2</td>
<td>104.2 (±2.25)</td>
<td>58.3-145.8</td>
</tr>
<tr>
<td>Ilomastat-IPC (6 mg/mL/600 Pa)</td>
<td>y = 6.70x + 17.43</td>
<td>17.43</td>
<td>6.7</td>
<td>0.930</td>
<td>4.9</td>
<td>7.8</td>
<td>109.0 (±2.47)</td>
<td>50-145.8</td>
</tr>
<tr>
<td>Ilomastat-IPC (3 mg/mL/Un-lyophilised)</td>
<td>y = 14.75x + 3.57</td>
<td>3.57</td>
<td>14.75</td>
<td>0.989</td>
<td>3.1</td>
<td>4.5</td>
<td>117.5 (±3.13)</td>
<td>50-141.7</td>
</tr>
<tr>
<td>Ilomastat-IPC (3 mg/mL/ Lyophilised)</td>
<td>y = 13.76x + 7.48</td>
<td>7.48</td>
<td>13.76</td>
<td>0.976</td>
<td>3.1</td>
<td>4.5</td>
<td>102.4 (±3.23)</td>
<td>62.5-145.8</td>
</tr>
<tr>
<td>AZ 8955-IPC (6 mg/mL)</td>
<td>y = 6.63x + 19.72</td>
<td>19.72</td>
<td>6.63</td>
<td>0.900</td>
<td>4.6</td>
<td>7.6</td>
<td>102.7 (±9.5)</td>
<td>53.8-145.8</td>
</tr>
<tr>
<td>AZ 8955-IPC (200 µL/ 6 mg/mL)</td>
<td>y = 3.71x + 14.82</td>
<td>14.82</td>
<td>3.71</td>
<td>0.953</td>
<td>9.4</td>
<td>14.9</td>
<td>106.0 (±2.67)</td>
<td>50-145.8</td>
</tr>
<tr>
<td>Pirfenidone-IPC (6 mg/mL)</td>
<td>y = 42.1x + 26.38</td>
<td>26.38</td>
<td>42.08</td>
<td>0.712</td>
<td>-</td>
<td>-</td>
<td>101.7 (±4.58)</td>
<td>52.5-137.5</td>
</tr>
<tr>
<td>Pirfenidone-only (6 mg/mL)</td>
<td>y = 38.7x + 29.02</td>
<td>29.02</td>
<td>38.72</td>
<td>0.745</td>
<td>-</td>
<td>-</td>
<td>100 (±5.6)</td>
<td>60-145.8</td>
</tr>
<tr>
<td>Pirfenidone-PLGA-IPC (2.8 mg/mL)</td>
<td>y = 18.52x + 9.41</td>
<td>9.41</td>
<td>18.52</td>
<td>0.647</td>
<td>-</td>
<td>-</td>
<td>93.75 (±4.2)</td>
<td>55-145.8</td>
</tr>
<tr>
<td>Pirfenidone-IPC (2.8 mg/mL)</td>
<td>y = 42.91x + 26.1</td>
<td>42.91</td>
<td>26.08</td>
<td>0.775</td>
<td>-</td>
<td>-</td>
<td>101.3 (±5.42)</td>
<td>50-141.7</td>
</tr>
<tr>
<td>Pirfenidone-IPC (20 mg/mL)</td>
<td>y = 23.42x + 41.2</td>
<td>23.42</td>
<td>41.22</td>
<td>0.656</td>
<td>-</td>
<td>-</td>
<td>106.8 (±4.0)</td>
<td>75-145.8</td>
</tr>
</tbody>
</table>
4.3.5 Pirfenidone-PLGA Microparticle Formulation

Due to the aqueous solubility of pirfenidone (20 mg/mL) it was encapsulated into polymer microparticles to attempt to slow drug release. Poly(lactic-co-glycolic) acid (PLGA) which is a hydrophobic copolymer linked together by ester bonds (Figure 80). PLGA has been used clinically and is broadly biocompatible, biodegradable and is used in many clinically approved formulations. Micro- and nano- drug particles of PLGA can be prepared to prolong drug release through a polymer matrix. Drug release occurs due water imbibition bulk degradation and resorption the polymer [253]. The PLGA that will be used in the study is 17 kDa with 50:50 ratio of LA: GA. This PLGA is reported to have a degradation time of 1 month [254] which is ideal for a 2 week release profile.

![Figure 80. The chemical structure of PLGA polymer. The x and y indicate the number of times each unit repeats.](image)

The pirfenidone loaded PLGA-drug particles were fabricated by an electrohydrodynamic process called electrospraying, which can generate high yields of micro- and nanoparticles with a narrow size distribution on a lab scale. Electrospraying is a one-step fibre and particle fabrication process [255]. The process is represented in Figure 81. The process relies on the ability of an electric field to deform the interface of a liquid droplet. When an electric field is applied to a droplet, a Coulombic force is generated within the droplet. Droplet breakup occurs when the applied Coulomb force overcomes the droplet cohesive forces within the droplet. This phenomenon starts at the shrinkage of the unstable, charged macro-droplet due to solvent evaporation. From the shrunk droplets smaller charged droplets are ejected as soon as the
surface tension is overcome by the Coulomb force. The droplets then form dry particles by the time they reach the collector [256].

The electrospraying configuration (Figure 81) is composed of a syringe pump, metal nozzle, high voltage power supply and a grounded substrate (particle collector). The syringe pump dispenses the solution at a controlled rate. The metal nozzle is connected to a high voltage power supply. The solution viscosity, solvent, and polymer properties and concentration affect the outcome of the electrospraying process. Depending on the polymer and the drug physicochemical properties these parameters are varied to achieve the desired particle shape and size [257]. To find the optimal electrospraying parameters, the voltage was varied from 14-22 kV, the flow rate 0.4-0.5 mL/min and the distance was varied from 15-20 cm. The electrospraying solution composition was also optimised. The polymer concentration in the solution was varied from 6-8% and the final drug loading was varied from 15-30%. This allowed the preparation of spherical microparticles with a size 1 µm in diameter.

![Figure 81. A schematic representation of the mechanism and configuration of electrospraying. Adapted from Nguyen, Clasen, and Van den Mooter 2016 [257].](image-url)
4.3.5.1 Physical Characterisation

To ensure spherical particle formation with the target particle size was successful achieved, SEM images were obtained of the electrosprayed sample followed by particles size analysis using the ImageJ software. The target average particle size was 1 µm to ensure that all particles would be fully entrapped inside the IPC gel. The particles were spherical (Figure 82) in shape and had an average diameter of 1.2 µm (±0.083). The SEM images showed that some particles were stuck to each other and none showed any visible pores. The particles did not show a hollowing or fibre formation. The chromatographic determination of pirfenidone drug content showed that the particle drug loading was 14% (± 0.5) which was 1% less than the target loading (15%).

![Figure 82. The particle size distribution and SEM image of the pirfenidone microparticles. 1) Particles size distribution graph, 2) SEM image of the pirfenidone encapsulated PLGA microparticles. Average diameter= 1.2 µm.](image)

4.3.5.2 Physicochemical Characterisation

FTIR spectroscopy was used to characterise the chemical functional groups of the pirfenidone-PLGA particles (Figure 83). The spectra of pirfenidone-PLGA particles displayed diagnostic infrared peaks found in PLGA and pirfenidone separately. The peaks at 1170, 1390, 1426, 1752 and 2954 cm⁻¹ which were only apparent in the PLGA spectra and not pirfenidone appeared in the spectra which combines both chemical products (pirfenidone-PLGA...
microparticles). Peaks at 1668 and 1268 cm$^{-1}$ which were only apparent in the pirfenidone spectra appeared in the spectra of pirfenidone-PLGA microparticles.

In the PLGA: the stretch at 1170 cm$^{-1}$ belonged to the C-O bond, the 1752 cm$^{-1}$ belonged to the C=O bond and the stretch at 2594 cm$^{-1}$ belonged to the O-H bond. For the pirfenidone molecule: the stretch at 1668 cm$^{-1}$ belonged to the C=C bond, the 1268 cm$^{-1}$ stretch belonged to the C-N bond and the stretch at 3048 cm$^{-1}$ belonged to the O-H bond.

![FTIR Spectra](image)

**Figure 83.** The difference in the FTIR spectra of pirfenidone, PLGA and PLGA-pirfenidone microparticles. The samples were analysed in the solid state at an ambient temperature.

XRD spectroscopy was conducted to investigate the effect of the electropspraying process on the pirfenidone, PLGA and pirfenidone-PLGA solid characteristics and compare the phase differences (Figure 84). Pifrenidone alone displayed crystallinity as indicated by the X-ray diffraction patterns which appeared as distinct peaks at different angles. The reflections of pirfenidone were observed in the range of 5°–80° at a diffraction angle of 2$\theta$ at 9.02°, 14.48°, 15.02°, 18.6°, 21.32°, 22.94°, 27.36°, 32.7° and 77.78°.
Both the PLGA only and the electrosprayed pirfenidone-PLGA particles showed an amorphous structure. The absence of a long range order (in amorphous solids) was indicated by the appearance of the halo XRD pattern.

DSC was conducted to evaluate the effect of the electrospraying process on the pirfenidone, PLGA and pirfenidone-PLGA thermal properties (Figure 85). Pirfenidone showed a sharp melting (endothermic) peak at 110.0°C. PLGA displayed glass transition temperature (T_g) of 41.9°C which was close to the reported literature value of 40°C [258]. There was a broad shallow endothermic transition at 46.7°C which represented the molecular relaxation of PLGA during the glass transition stage [259]. As a polymer PLGA would be expected to have an amorphous structure, as confirmed by the XRD spectra (Figure 83), and no crystallisation peak for PLGA was observed in the DSC. The melting peak of pirfenidone disappeared in the PLGA-pirfenidone particles indicating that pirfenidone was amorphous in the particles which was

Figure 84. The difference in the XRD spectra of PLGA, pirfenidone and PLGA-pirfenidone microparticles. Pirfenidone shows a crystalline structure but the other materials show an amorphous structure. The samples were analysed in the solid state.
also confirmed by halo in the XRD spectra. The pirfenidone-PLGA particles displayed a $T_g$ which was shifted to the right and had a value of around $62.8^\circ\text{C}$. The molecular of PLGA relaxation was also shifted to the right to a value of $71.0^\circ\text{C}$.
Figure 85. The DSC thermogram of the pirfenidone-IPC formulation and its components. Pirfenidone $T_m=$ melting point $= 110^\circ$C, PLGA $T_g=$ glass transition $= 41.9^\circ$C, pirfenidone-PLGA $T_g= 62.75^\circ$C. All samples were analysed in the solid state.
4.3.5.3 In-vitro Drug Release

The effect of encapsulating pirfenidone into a PLGA particle on drug release from the IPC formulation is shown Figure 86. The particle formulation contained 20 mg/mL of particles with a 2.8 mg/mL pirfenidone content (20 mg particles/mL IPC). The particle drug loading was 14%. The pirfenidone IPC formulation also contained 2.8 mg/mL of drug. The pirfenidone only IPC formulation released the drug over 2 days whereas the pirfenidone-PLGA microparticle containing IPC released the drug in 4 days. The pirfenidone-PLGA-IPC formulation had no significant effect (p= 0.137) on the concentration profile compared to the pirfenidone-IPC formulation. Both formulations had a similarly high burst release (~20%) and released over 80% of the drug on day 1. Particle loading had a significant effect (p= 0.03) on the cumulative drug release. For example, on day 1 the drug concentration released was 86 µg/mL and 71.7 µg/mL for the IPC-pirfenidone and IPC-pirfenidone-PLGA particles respectively.

Figure 86. The change in the in-vitro drug release from PLGA encapsulated pirfenidone microparticles in IPC vs pirfenidone-IPC formulations, at 37°C. The drug loading was 2.8 mg/mL in a 100 µL depot. The values represent the mean± SE and at n=3. Cumulative release: p= 0.03 and Drug concentration: p= 0.137 when the data from the two conditions were compared using F-ANOVA. *= Statistically significant (p < 0.05). N/S= Not statistically significant (p > 0.05).
4.3.6 Biological Characterisation

4.3.6.1 Effect of DMSO on Cell Viability

Dimethyl sulfoxide (DMSO) is used widely to as a cryoprotectant to preserve cells, it is still potent and has shown cytotoxicity in some cell lines at some concentrations \(^\text{[260]}\). DMSO cytotoxicity was evaluated on HTF cells \textit{in-vitro} to establish what concentrations of DMSO could be used to formulate IPC drug formulations. One of the formulation approaches aimed to prepare drug solutions then precipitate into microparticles as they are mixed with the IPC solution. This could be achieved by solubilising a high concentration of the drugs in a solution of DMSO and EDTA which then is mixed with the IPC. The particle precipitate would form as the DMSO concentration is diluted by the IPC. DMSO is a good solvent as the molecule is amphipathic which can solubilise a wide range of molecules \(^\text{[261]}\).

DMSO concentration had a significant effect on the cell viability \((p<0.001)\) (\textit{Figure 87}). At 1% DMSO the cell viability remained very high (\(>90\%\)) at all time points \((p=0.877)\). At 5% DMSO, the cell viability was above 90% at day 1 but dropped significantly with time. There was no significant difference \((p=0.881)\) between 1% and 5% DMSO on day 1. At 10% DMSO reduced cell viability on day 1 and increased with time. On day 3 for 1, 5 and 10% DMSO, the cell viability was 94.5, 41.5 and 29.4% respectively. Therefore, DMSO was an unsuitable solvent for use in IPC formulations and was as a result not used to formulate the drugs.
4.3.6.2 In-vitro Effect of Anti-scarring Drugs on HTF Cell Viability

Ilomastat and AZ 8955 have shown promising anti-scarring properties when used in a rabbit model of glaucoma filtration surgery to improve bleb survival [133], [229]. The effect of the three drugs on human tenon fibroblasts (HTF) cell viability was evaluated in-vitro. These cells were ideal to utilise as they are commonly found in the subconjunctiva and are involved in trichiasis pathology and are a major drug target [129]. A comparison of the local effect of the different concentrations of the drugs on HTF cell viability is therefore important. The non-sink conditions in-vivo meant that the maximum concentration of drug to which tissues are exposed would be at its solubility limit therefore the drugs were compared at their solubility limit. The cell viability effect of samples released from AZ 8955-IPC and ilomastat-IPC formulations on day 7 was evaluated to establish whether the drug properties change with time.

For pirfenidone, the cell viability (Figure 88) remained high (> 90%) up to 500 µg/mL of pirfenidone. Above 500 µg/mL dropped significantly to around 1.5% at 5 - 20 mg/mL at all time points. Even though the cell viability profile

Figure 87. The effect of DMSO concentration and incubation time on the in-vitro HTF cell viability. Cells incubated in 1, 5 and 10% DMSO in complete DMEM and for 1, 2 and 3 days at 37°C and 5% CO₂. The values represent the mean± SE and at n=3. p values < 0.001 when the cell viability data in different DMSO concentrations were compared at each day using ORM-ANOVA. * = Statistically significant (p < 0.05).
seemed to drop at the 48-h time point, there was no significant effect on cell viability (p= 0.179). The IC\textsubscript{50} values on day 1, 2 and 3 were 2.54, 2.5 and 2.57 mg/mL respectively.

**Figure 88.** The change in the in-vitro cell viability of HTF cells incubated in different pirfenidone concentrations for 24, 48 and 72 h at 37°C and 5% CO\textsubscript{2}. The values represent the mean± SE and at n=5. p= 0.179 when the different incubation time profiles were analysed using F-ANOVA. N/S = Not statistically significant (p > 0.05).

The cell viability of HTF cells in different concentrations of ilomastat on day 1, 2 and 3 is shown in **Figure 89.** The cell viability in ilomastat remained very high (above 88%) for all the evaluated concentrations at all three incubation times. At certain concentrations the cell viability increased above 100%. It was 114% of the control at 50 µg/mL. Time had a significant effect (p= 0.023) on the cell viability profile of HTF cells which was indicated by the higher cell viability at 72 h.

The cell viability in ilomastat released from IPC gel on day 7 was similar to that of the freshly prepared drug at the same concentration (p= 0.755). At 72 h and a drug concentration of 12.5 µg/L, the cell viability was was 109.2% and 106.9% for released and freshly prepared ilomastat respectively (**Figure 89**).
The effect of AZ 8955 on the cell viability profile of HTF cells (Figure 90) showed a concentration dependent profile. Time did not have a statistically significant effect \((p=0.517)\) on the HTF cell viability profile. The cell viability of HTF cells was seen to slowly decrease with the increase in drug concentration. At 140 \(\mu\text{g/mL}\), the cell viability profile at 72 h appeared higher (75%) than at 24 h (67%) and 48 h (64%).

The 72 h HTF cell viability (Figure 89) of HTF cells incubated with AZ 8955 (12.5 \(\mu\text{g/mL}\)) released from the IPC formulation was similar to that of the freshly prepared drug and was not significantly different \((p=0.721)\).

**Figure 89.** The change in the in-vitro cell viability of HTF cells incubated in different ilomastat concentrations for 24, 48 and 72 h and formulated ilomastat (incubated for 72h) at 37°C and 5% CO\(_2\). The cell viability in formulated ilomastat (12.5 \(\mu\text{g/mL}\), released from the IPC formulation on day 7(\text{+})) was that of drug free control. The values represent the mean± SE and at \(n=5\). \(p=0.023\) when the three incubation time profiles were analysed using F-ANOVA. * = Statistically significant \((p < 0.05)\).
The cell viability of HTF cells at 72 h (Figure 91) for the different concentrations of anti-scarring drugs was plotted to compare the different drugs. The drugs had a significant effect (p< 0.001) on cell viability even when compared against each other within the same concentration range (up to 100 µg/mL). AZ 8955 caused the cell viability to drop to below 90% at concentrations above 50 µg/mL. Ilomastat, surprisingly, caused the cell viability to increase to above 100% at concentrations up to 100 µg/mL. Pirfenidone resulted in a much higher cell viability (100%) than AZ 8955 at concentrations up to 500 µg/mL and dropped significantly at higher concentrations. The cell viability in pirfenidone dropped significantly at higher concentrations indicating that the formulation release might need to be maintained below 1 mg/mL.

**Figure 90.** The change in the in-vitro cell viability of HTF cells incubated in different AZ 8955 concentrations for 24, 48 and 72 h and formulated AZ 8955 (incubated for 72h) at 37°C and 5% CO2. The cell viability in formulated AZ 8955 (12.5 µg/mL, released from the IPC formulation on day 7(†)) was of drug free control. The values represent the mean± SE and at n=5. p= 0.517 when the three incubation time profiles were analysed using F-ANOVA. N/S = Not statistically significant (p > 0.05).
At concentrations up to 100 µg/mL, the drug type had a significant effect on cell viability at 24 h (p=0.041) at 48 h (p = 0.035) and 72 h (p = 0.065). For all the experimental groups, drug concentration had a significant effect (p< 0.001, within-subject F-ANOVA) on HTF cell viability.

**Figure 91.** The in-vitro cell viability profile of HTF cells incubated for 72 h in different concentrations of ilomastat, AZ 8955 and pirfenidone solutions at 37°C and 5% CO₂. The values represent the mean± SE and at n=5. p< 0.001 when the different drug profiles were analysed using F-ANOVA. * = Statistically significant (p< 0.05).

### 4.3.6.3 In-vitro Cytotoxicity of IPC Formulation

To investigate whether the IPC formulation of AZ 8955 and ilomastat had any effect on the cell viability of HTF cells after 72 h. The ilomastat-IPC formulation and IPC only samples (Figure 92) did not show any cytotoxicity compared to the control (HTF cells only). The cell viability seen in the ilomastat formulation was similar (p= 0.341) to the cell viability in 140 µg/mL ilomastat (Figure 89). The AZ 8955-IPC formulation showed a reduction HTF cell viability which were similar to those seen in the drug only experiments at 140 µg/mL (p= 0.879) at 72 h (Figure 90). EDTA is used clinically in humans (>35 mM ) as a metal chelator in lead poisoning where it is given intravenously. EDTA can, however, be toxic to cells after a prolonged exposure. EDTA concentration was maintained below 1.88 mM in this study as the in-vitro experiment could
not replicate the continuous removal of EDTA from the area of injection in-vivo (see Chapter 5 for more details).

Figure 92. The in-vitro cell viability of HTF cells incubated with IPC, IPC-AZ 8955 and IPC-ilomastat formulations for 72 h at 37°C and 5% CO₂. The values represent the mean ± SE and at n=3. p< 0.001 when the different cell viability profiles were compared using ORM-ANOVA. * = Statistically significant (p < 0.05).

4.3.6.4 Contraction Assay of Anti-scarring Drugs

To compare the bioactivity of the drugs in terms of MMP inhibition, a contraction assay was used. To study whether the formulation or time affects drug activity, samples of released ilomastat and AZ 8955 from IPC on day 7 were evaluated. The assay was used in various wound healing studies [141], [236] and involves using fibroblast embedded collagen type I gels. A contraction assay allows for the investigation of the fibroblast-mediated contraction phase of wound healing [262], [263]. The migration of fibroblasts through the ECM and contraction of collagen embedded tissues is a fundamental process in wound healing and scarring [228].

The study was conduct over 7 days where the extent of gel contraction with different drug concentrations was visually monitored daily and then analysed using the ImageJ software [133], [138]. The rate of contraction was represented by the slope of the trend line for each experimental condition. The drug efficacy relative to other drugs was calculated by dividing the percentage of contraction at a certain time point at a specific concentration by that of the
other drug. The percentage of contraction was plotted as a percentage of gel diameter normalised to original diameter. To check that the change in gel contraction was entirely due MPP inhibition and not cell death, a live-dead imaging fluorescence-based assay was used on day 7 of the study.

Images (Figure 93) of the contraction assay showed that the positive control (in 10% BSA) did not show any contraction. The negative control (in 10% FBS) showed a significant contraction. Pirfenidone (250 µg/mL) treated cultures were slightly larger than the positive control. Collagen cultures treated with ilomastat (100 µg/mL) and AZ 8955 (50 µg/mL) resulted in less contracted collagen gels.

**Figure 93.** Sample images of HTF cells embedded in collagen gels under different conditions on day 7. Positive control (contracted drug free), negative control (drug free uncontracted), pirfenidone (250 µg/mL), ilomastat (100 µg/mL) and AZ 8955 (50 µg/mL).

The 7-day contraction profile (Figure 94) of HTF embedded collagen gels exposed to different concentrations of pirfenidone showed a time dependent increase. At 12.5-100 µg/mL concentrations, pirfenidone failed to cause any visible decrease in collagen contraction compared to the positive control. The drug concentration had a significant effect (p< 0.001, within-subject F-ANOVA) on the HTF-collagen contraction. Only the 250 µg/mL of pirfenidone showed a visible decrease in collagen contraction which became
apparent after day 2 compared to the control. There was a significant difference (p< 0.001) between the drug free control and 250 µg/mL pirfenidone cultures. Only the 250 µg/mL pirfenidone concentration showed a reduction in the contraction rate and a contraction inhibition level of 12.3% of the drug free control (Table 16).

**Figure 94.** The extent of the 7-day in-vitro contraction of HTF embedded collagen gels incubated with different pirfenidone concentrations compared to the drug free control at 37°C and 5% CO₂. Drug concentrations ranged from 12.5 - 250 µg/mL. The values represent the mean± SE and at n=3. p=0.01 when the different concentration profiles were compared using F-ANOVA. * = Statistically significant (p < 0.05).
**Table 16.** The effect of different concentrations of pirfenidone on the in-vitro contraction profile of HTF embedded collagen gels on day 7 of incubation at 37°C and 5% CO. Data extracted from the collagen gel contraction profiles, at n=3.

<table>
<thead>
<tr>
<th>Drug concentration (µg/mL)</th>
<th>Contraction level (%)</th>
<th>Contraction rate (%/day)</th>
<th>Inhibition level of the control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>67.3</td>
<td>9.5</td>
<td>-</td>
</tr>
<tr>
<td>12.5</td>
<td>67.8</td>
<td>10.1</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>65.7</td>
<td>9.9</td>
<td>3.0</td>
</tr>
<tr>
<td>50</td>
<td>65.7</td>
<td>9.0</td>
<td>3.0</td>
</tr>
<tr>
<td>100</td>
<td>65.1</td>
<td>9.8</td>
<td>3.9</td>
</tr>
<tr>
<td>250</td>
<td><strong>59.5</strong></td>
<td><strong>8.4</strong></td>
<td><strong>12.3</strong></td>
</tr>
</tbody>
</table>

To ensure that gel contraction was not due to any cytotoxicity, a live-dead staining assay (**Figure 95**) was conducted. Pirfenidone was non-toxic to the HTF cultures at all the evaluated concentrations (12.5-250 µg/mL). There were almost no dead cells (red areas) and almost all cells were viable (green areas). The significant gel contraction, distance between cells of drug containing HTF embedded collagen gels was high and similar to the drug free control. The morphology of the HTFs was normal and appeared fibroblast-like (elongated).
The bioactivity (Figure 96) of ilomastat in terms ECM remodelling (gel contraction) was higher than that of pirfenidone. The gel contraction increased continuously over time for all the experimental conditions. The gel contraction was reduced by the presence of ilomastat. The extent of collagen contraction
was reduced by the increase in drug concentration. The ilomastat concentration had a statistically significant effect (p< 0.001, within-subject F-ANOVA) on the collagen contraction profile. Drug concentration had no significant effect (p= 0.052) when comparing the 25 µg/mL to 50 µg/mL profiles. Formulated ilomastat (12.5 µg/mL) released from the IPC formulation on day 7 showed a similar ECM remodelling bioactivity (Figure 96) to the freshly prepared ilomastat at the same concentration (p= 0.150).

When different drug concentrations were compared on day 7 (Table 17) the collagen was inhibited as seen by the decrease in the extent of collagen contraction. The reduction was significant but not proportion to the increase in concentration. To compare the effect of different concentration to the drug free (positive) control a percentage of level of inhibition was established (Table 17). It was shown that the increase in ilomastat concentration from 50 to 100 µg/mL had the most pronounced effect. The collagen contraction rate at 100 µg/mL was more than 3x lower compared to the control. For the formulation, the rate of collagen contraction in formulated ilomastat was the same as in the freshly dissolved ilomastat (6.3%/day).
Table 17. The effect of different concentrations of ilomastat on the in-vitro contraction profile of HTF embedded collagen gels on day 7 of incubation at 37°C and 5% CO₂. Data extracted from the collagen gel contraction profiles, at n=3.

<table>
<thead>
<tr>
<th>Drug concentration (µg/mL)</th>
<th>Contraction level (%)</th>
<th>Contraction rate (%/day)</th>
<th>Inhibition level of the control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>70.8</td>
<td>10.1</td>
<td>-</td>
</tr>
<tr>
<td>12.5</td>
<td>46.1</td>
<td>6.3</td>
<td>35</td>
</tr>
<tr>
<td>25</td>
<td>35.9</td>
<td>5.6</td>
<td>44</td>
</tr>
<tr>
<td>50</td>
<td>39.5</td>
<td>5.1</td>
<td>49</td>
</tr>
<tr>
<td>100</td>
<td>25.3</td>
<td>3.2</td>
<td>64</td>
</tr>
</tbody>
</table>

The live-dead staining assay (Figure 97) showed that ilomastat had no cytotoxicity (very few red areas) at all evaluated concentrations (12.5-100 µg/mL). Instead there was a high number of fluorescent green areas (live cells) and the HTF cells showed a typically healthy fibroblast elongated morphology. The HTF embedded collagen gel which contained 0 µg/mL showed a higher cell density than the drug containing gels. This was due to the cells being much...
closer to each other due to the significant contraction of the collagen gel where the same number of cells occupied a smaller area.

**Figure 97.** Live-Dead staining of HTF cells embedded in collagen gels, incubated in different ilomastat concentrations and imaged on day 7 of the contraction assay study. The positive control was drug free HTF cultures. Cultures were incubated at 37°C and 5% CO₂. Live cells (Calcein AM, green), and Dead cells (DNA dye, red).
The inhibition of collagen contraction by AZ 8955 (Figure 99) was increased with increase drug concentration. It was noted that collagen contraction increased with time for the experimental conditions apart from the 25 and 50 µg/mL of AZ 8955. Collagen contraction increased up to day 3 and then remained relatively constant at these two drug concentrations. The effect of drug concentration on the extent and rate of collagen contraction became pronounced after day 2. Released AZ 8955 (12.5 µg/mL) from the IPC formulation on day 7 showed a similar contraction pattern (Figure 98) to the freshly prepared drug. The level of gel contraction on day 7 was 32.2% and 33.2% for the released and the freshly prepared drug respectively. The change in AZ 8955 condition did not show any significant effect (p= 0.992) on the extent of collagen contraction.

Figure 98. The change in in-vitro contraction of HTF embedded collagen gels over 7 days in different concentrations of freshly prepared AZ 8955 and formulated AZ 8955. Drug concentrations ranged from 12.5 - 100 µg/mL. Formulated AZ 8955 (12.5 µg/mL) was released from the IPC formulation on day 7. Samples were incubated at 37°C and 5% CO₂. The values represent the mean± SE and n=3. p< 0.001 when the different concentration profiles of the freshly prepared AZ 8955 where compared using F-ANOVA. * = Statistically significant (p < 0.05).
On day 7, the contraction profiles at different drug concentrations were compared (Table 18). AZ 8955 caused a more significant reduction in the collagen gel contraction than ilomastat at the same concentrations. The rate of gel contraction decreased with the increase in drug concentration. The contraction rate decreased by more than 6× when comparing 50 µg/mL AZ 8955 to the control and showed an 82% inhibition of the control. The contraction rate in the formulated AZ 8955 was similar (5.1%/day) to the freshly prepared drug (5.4%/day).

Table 18. The effect of different concentrations of AZ 8955 on the in-vitro contraction profile of HTF embedded collagen gels on day 7 of incubation at 37°C and 5% CO. Data extracted from the collagen gel contraction profiles, at n=3.

<table>
<thead>
<tr>
<th>Drug concentration (µg/mL)</th>
<th>Contraction level (%)</th>
<th>Contraction rate (%/day)</th>
<th>Inhibition level of the control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>67.6</td>
<td>9.8</td>
<td>-</td>
</tr>
<tr>
<td>12.5</td>
<td>37.5</td>
<td>5.4</td>
<td>44.0</td>
</tr>
<tr>
<td>25</td>
<td>18.0</td>
<td>2.5</td>
<td>73.0</td>
</tr>
<tr>
<td>50</td>
<td>11.8</td>
<td>1.7</td>
<td>82.0</td>
</tr>
</tbody>
</table>

The cell viability of HTF on day 7 (Figure 99) with different concentrations of AZ 8955 did not display any cytotoxicity. The images showed mostly green fluorescent (live) cells and almost no red cells (dead) cells. At 0 µg/mL drug concentration (control) HTF cells appeared much closer to each other than drug treated cell cultures. This was due to the significantly higher extent of gel contraction which brought the cells much closer together. A significantly lower level of gel contraction at 50 µg/mL resulted in much more sparse cells. In both control and drug treated samples the HTF cells maintained their fibroblast like morphology of an elongated shape.
4.3.6.4.1 Summary of the Contraction Assay Profiles

The effect of the different drugs on collagen contraction for a 7-day period (Figure 100) was analysed. At all drug concentrations, AZ 8955 caused a greater inhibition in collagen contraction than ilomastat which was apparent at concentrations above 12.5 µg/mL. AZ 8955 and ilomastat were much more effective at inhibiting collagen contraction than pirfenidone at all concentrations. The contraction levels on day 7 were compared to analyse the efficacy of the different drugs (Table 18). The efficacy was calculated by dividing the contraction values of the drugs at a certain concentration. AZ 8955 was the most efficacious drug, and at 50 µg/mL it was 5.6× and 3× more...
efficacious than pirfenidone and ilomastat respectively. Ilomastat was 1.8× more efficacious than pirfenidone at 50 µg/mL.

Table 19. The difference in contraction efficacy of AZ 8955, ilomastat and pirfenidone on day 7, at n=3. The contraction assay of HTF cells embedded in collagen gels and incubated with different drug concentrations at 37°C and 5% CO₂ for 7 days. Efficacy was defined as the ratio of the contractions at different drug concentrations on day.

<table>
<thead>
<tr>
<th>Drugs (A:B)</th>
<th>Drug concentration (µg/mL)</th>
<th>Gel contraction (%)</th>
<th>Efficacy (A/B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ilomastat: Pirfenidone</td>
<td>100:250</td>
<td>25.3: 59.5</td>
<td>2.3</td>
</tr>
<tr>
<td>Ilomastat: Pirfenidone</td>
<td>100:100</td>
<td>25.3: 65.1</td>
<td>2.6</td>
</tr>
<tr>
<td>Ilomastat: Pirfenidone</td>
<td>50:50</td>
<td>39.5: 65.7</td>
<td>1.8</td>
</tr>
<tr>
<td>AZ 8955: Pirfenidone</td>
<td>50:250</td>
<td>11.8:59.5</td>
<td>5.0</td>
</tr>
<tr>
<td>AZ 8955: Pirfenidone</td>
<td>50:50</td>
<td>11.8: 65.7</td>
<td>5.6</td>
</tr>
<tr>
<td>AZ 8955: Ilomastat</td>
<td>50:100</td>
<td>11.8: 25.3</td>
<td>2.1</td>
</tr>
<tr>
<td>AZ 8955: Ilomastat</td>
<td>50:50</td>
<td>11.8: 39.5</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Figure 100. The in-vitro contraction levels of HTF embedded collagen gels in different concentrations of AZ 8955, pirfenidone and ilomastat on day 7. Samples were incubated at 37°C and 5% CO₂ for 7 days. The values represent the mean± SE and at n=3. p values < 0.001 when the contraction profiles of different drugs were compared at the same concentrations using ORM-ANOVA. * = Statistically significant (p < 0.05).
4.3.7 MMP Activity Assay

The level of MMP inhibition by the IPC formulations of ilomastat and AZ 8955 was evaluated on samples (12.5 µg/mL drug concentration) released on day 7 (Figure 101). Media was collected from the contraction assay experiments and analysed at different time points. A higher fluorescence unit reading indicated a higher MMP activity. There was a significant MMP inhibition (p< 0.001) by both formulations compared to the control (drug free HTF cells embedded collagen gel). The drug free control showed an increase in MMP activity with time (p< 0.001). The ilomastat-IPC and AZ 8955-IPC formulations (at 12.5 µg/mL drug concentration) showed no change in MMP inhibition with time for the ilomastat-IPC (p= 0.898) and AZ 8955-IPC formulation (p= 0.978). The inhibition profile of the IPC formulations was similar to the control in both AZ 8955 (p= 0.443) and ilomastat (p= 0.703). The control was media collected from collagen gel embedded HTF cells exposed to 12.5 µg/mL freshly dissolved drug. Both formulations AZ 8955 and ilomastat remained equally active to the control after 7 days in the formulations. The level of MMP inhibition caused by both formulations did not differ significantly (p= 0.247).

![Figure 101](image_url)

**Figure 101.** The effect of formulated and freshly prepared AZ 8955 and ilomastat on MMP enzyme activity levels (FU levels) at different time points in HTF cells embedded in collagen gels. Levels were measured from the contraction assay samples on day 0, 3 and 7 where the Control = drug free gel. The values represent the mean± SE and at n=3. p values < 0.001 when the drug containing samples on each day were compared to the control by using ORM-ANOVA. * = Statistically significant (p < 0.05).
4.3.8 Summary of Results

An anti-scarring IPC formulation of AZ 8955 and ilomastat was successfully prepared. A 100 µL depot of AZ 8955 and ilomastat IPC formulations with a drug loading of 6 mg/mL achieved a 2-week slow release profile. The particle size of both AZ 8955 and ilomastat IPC formulations was ~7 µm in size at 6 mg/mL drug loading. Pirfenidone IPC formulations (drug only and PLGA encapsulated) failed to achieve a slow release profile. Lyophilisation did not affect drug release from the IPC formulation. Drug release was depot size and drug loading dependent. No drug degradation was observed throughout the release study, indicated by the lack of degradation peaks in HPLC. Over 96% of the loaded drug was released from all three IPC formulations.

Pirfenidone showed significant of reduction in cell viability at concentrations above 1 mg/mL. ilomastat showed a cell protective effect by increasing the cell viability compared the control in concentrations up to 100 µg /mL. AZ 8955 resulted in some levels of reduction in cell viability at concentrations above 50 µg/mL. When the drugs where compared in terms of their effect on collagen contraction (ECM remodelling) pirfenidone showed very little effect on collagen contraction. Ilomastat and AZ 8955 showed significant levels of inhibition in collagen contraction where AZ 8955 was order of magnitude more efficacious at inhibiting collagen contraction than ilomastat.

When ilomastat-IPC and AZ 8955-IPC formulations were biologically evaluated they showed reduction of cell viability of HTF cells. The AZ 8955-IPC formulation showed similar cell viability. When samples released from the formulations on day 7 where evaluated, they did not show any change in the cell viability profile or bioactivity (collagen contraction) compared to the freshly dissolved drugs at the same concentration. The IPC formulations of AZ 8955 and ilomastat also showed a similar MMP inhibition profile to the control when samples released on day 7 were evaluated.
4.4 Discussion

There are several advantages to powdered gel formulation which include reduction in risk of drug degradation as the drug is solid form. Ilomastat has actually been shown to degrade when stored in solutions [133] The poor drug solubility allows a higher drug loading into IPC which can exploit the non-sink conditions of the subconjunctival space. A powdered gel IPC formulation also ensures the localisation of the drug deposition and minimises the foreign body reaction that is expected to be favoured at a site of surgery.

Different drug mixtures to IPC solution ratios were evaluated to achieve a homogenously mixed formulation without reducing the final mechanical strength of the gel. A 1:10 ratio of drug mixture: IPC solution was the most ideal both in terms of mixing time and its effect on the gel mechanical properties (section 3.3.4.3 in Chapter 3). This therefore meant that the drug concentration in the drug suspension was 10 times more concentrated to achieve the required final concentration.

Mixing of viscous fluids such as IPC created air bubbles which were difficult to remove. Several methods were evaluated including centrifuging and the most ideal method which was to use a pressure polymerisation unit. The device applies high pressure to remove air bubbles [264]. This process is time consuming and complete removal of all microscopic air bubbles cannot be guaranteed. A potentially time saving method which also avoids the formation of air bubbles all together and reduce transfer losses is the FlackTek Speed Mixer (FlackTek Inc, US). It can be used to mix the IPC solution and the drug mixture and is based on dual asymmetric centrifugal mixing. The mixing cup in the device undergoes double rotation where a combination of centrifugal forces acting at different levels enable rapid mixing of the entire cup. It has also been used to mix solids, powders, liquids and creams, in any combination. However, the minimum amount used is 1 g and the device is costly [265].

Ilomastat and AZ 8955 were both in powder form as microparticles (~7 µm in size on average) (Figure 64) inside the gel. Viscous fluids such as the IPC formulations can shear thin under stress of for example injection through a small gauge needle and then self-heal after the stress is removed. Some fluids show a very slow recovery due to disruption of the non-specific
interactions which they rely on \cite{266}. The final mechanical strength (Figure 66) of the gel formulation was not affected by injection of the IPC-drug solution though a 26G needle. The presence of microparticles in the IPC and the subsequent needle extrusion did not affect the integrity of the collagen in the hydrogel.

Alvarez et al reported the mechanical strength of collagen hydrogels embedded with drug encapsulated silica nanoparticles depended on the particles size. With 500 nm particles size there was a higher interaction between the collagen fibrils resulting in a denser fibril denisty and hence higher mechanical stiffness. The 100 nm particles reduced the interactions and therefore reduced the mechanical strength of the gel. They described this as being caused by the small particles (e.g 100 nm) becoming adsorbed on the fibril surface while the larger particles were coated by a converging assembly of fibrils \cite{267}. Therefore, particle size might have an effect on the gel mechanical properties.

The drugs remained crystalline inside the formulation even when freeze-dried which was confirmed by the DSC spectra of ilomastat-IPC (Figure 69) and AZ 8955-IPC (Figure 70) formulations. There was melting peak at 210°C for ilomastat and at 140°C for AZ 8955 in the IPC formulations. These melting peaks were strongly visible in the in the DSC thermogram of the drugs alone and matched the reported literature values \cite{240}. The thermal denaturation point (122°C) of collagen in IPC was very close to the literature value (126°C). However, the thermal denaturation point of collagen in the IPC solution might be higher than the than the literature value (42°C) due to the presence of EDTA \cite{105}, mannitol and sucrose \cite{196,197}.

The IPC did not have an effect on the drug release (Figure 72) indicating that none of the drugs interacted with the collagen. The interaction of drug with the gel could potentially allow for a slower drug release. The fact that the mesh size was up to 300 nm meant that low molecular weight moelcules like pirfenidone could easily flow out without hinderance. Ilomastat and AZ 8955 were also formulated as solutions with IPC (data not shown) at low loading. This was done by incorporating the drugs as solutions into the IPC at a final loading below their water solubility (140 µg/mL). All drug formulations released the drug within 2 days.
An affinity-based growth factor delivery system was developed where interaction of the drug with the delivery system prolonged drug release\textsuperscript{[268]}. This system utilised a photochemically crosslinked heparin-alginate which contained bone morphogenetic protein-2 (BMP-2) which a protein drug. The hydrogel formulation was prepared by photopolymerisation of methacrylated alginate and methacrylated heparin in the presence of BMP-2 to try to ensure the protein was within the crosslinked polymer network. The alginate crosslinks and heparin linkages contain ester groups that are hydrolytically labile to ensure hydrogel degradability. The BMP-2 was able to bind to the heparin via affinity binding, i.e. electrostatic interactions where the positively charged sulphate group in heparin interacted with the positively charged amino acids in the proteins. Drug release then occurred through disassociation from heparin and through hydrolytic degradation of the gel crosslinks. The 6 mm hydrogel implant showed a slow drug release profile and maintained the bioactivity of the BMP-2. \textit{In-vivo} it showed ectopic bone formation when implanted into immunodeficient mice\textsuperscript{[268]}. Due to a high gel mechanical stiffness (~200 kPa) surgical implantation was necessary. The target location of the implant can be potentially an issue\textsuperscript{[268]}. According to Nair and Jacob, in humans, a 6 mm implant in mice would potentially be 146 cm in diameter. Further optimisation of the formulation is required to reduce the implant size\textsuperscript{[269]}. The presence of any unreacted methacrylate needs to be evaluated as it can cause cytotoxicity\textsuperscript{[270]}. Testing in immune deficient mice meant that the monitoring of the immune response to the implant was not possible\textsuperscript{[271]}.

Another potential affinity-based drug delivery using the IPC may possibly be developed using a collagen binding peptidoglycan peptide. A peptide showed very good anti-scarring properties with no cytotoxicity and can potentially interact with collagen in IPC and causing a slowdown in its release from the gel\textsuperscript{[272]}.

When IPC was formulated using a powdered gel formulation of AZ 8955 and ilomastat, the drug release (\textit{Figure 74}) was prolonged and controlled by the solubility limit of the two drugs under non-sink conditions. A 100 µL depot with 6 mg/mL of drug had a 2-week release profile. The drug release was similar for both AZ 8955 and ilomastat which could be due to their similar water solubility. Drug release was dependent on the drug concentration (\textit{Figure 76})
and depot size (Figure 77). This meant that the same amount of media flowing across the formulation would flow across larger amount of drug. These parameters would likely have a similar effect on the in-vivo release profile as flow of tissue fluids inside the subconjunctiva is relatively constant [240]. The release kinetic profile of the formulation could be described as zero order ($R^2 \geq 0.9$). The release profiles did not fit other release kinetic models such as Higuchi’s and Hixson-Crowell [273]. The release rate changed with the increase in drug loading and implant size. This was likely due to a variation in flow rate between experiments, different particle distribution through the gel and incomplete removal of all microscopic air bubbles which could result in variation in depot size and particle distribution. Pipetting the IPC formulation into the release chamber resulted in implants with a slightly different shapes and different surface area to volume ratio which may affect the drug release as described by Reynolds, Mitchell, and Balwinski [274].

Drug release in the powdered gel formulations for ilomastat and AZ 8955 can be described by the dissolution of the drug from the microparticles. During the dissolution process the surface of the drug particles is first wetted by water followed by the molecules dissolving of the surface. Then, the molecules diffuse out of the gel and are removed by the flow of media. Therefore, the rate of dissolution and hence the implant drug release can also be described by Noyes-Whitney equation. This means that the drug release is controlled by the rate of media flow, the surface area of the particles as well as the dose [275].

Parkinson et al prepared 1 mg tablets of ilomastat by direct compression without the use of excipients. These were prepared for bleb survival after a glaucoma filtration surgery. The tablet had a slow release drug profile and the drug remained the stable over the release period [240]. However, a local foreign body response was an issue reported later by the formation a fibrotic capsule around the tablet implant in the rabbit subconjunctiva [133]. Hence the need for an IPC formulation.

Water has a hydrolytic effect on certain covalent bonds and many drugs are unstable in water solutions. Chemical degradation is a critical aspect in the stability of pharmaceuticals. The rate and extent of hydrolysis of drugs in aqueous solutions depends on several parameters. Ilomastat has been shown
to degrade in aqueous solution with time. These include the type and numbers of susceptible functional groups (e.g. esters), pH and temperature. The formulation was lyophilised to ensure long term stability. The in-vitro release data was promising and showed very similar release profiles for both lyophilised and non-lyophilised formulations (Figure 78). Drug release was not affected by the lyophilisation process and remained relatively the same as the un-lyophilised formulation.

Pirfenidone was formulated into PLGA microparticles to slow its release down and reduce the effect of high concentration on released on HTF cells. The moderate water solubility of pirfenidone (20 mg/mL) meant that it was released very quickly from the gel as the drug was dispersed throughout the system. Pirfenidone-PLGA microparticles were fabricated by electrospraying to avoid the need of emulsions and difficulty to remove residual solvents. Drug release was prolonged to 4 days (Figure 86) due to the time taken for water to penetrate and hydrate the PLGA particles. When AZ 8955 was formulated into PLGA microparticles at a similar particular size and loading level, the release profile was still rapid and did not change. Several factors could potentially be the cause including that electrospraying charges the solution and the drug in the process resulting in the drug potentially being distributed at the surface of the particles. The particles had a high surface area to volume ratio which meant a faster wetting time and hence drug release.

The electrosprayed pirfenidone-PLGA particles (14% drug loading) display no degree of crystallinity and appeared to be completely amorphous as shown by the XRD spectra by the presence of a halo (Figure 84) and the DSC thermogram by the presence of T_g (Figure 85). This matched the literature reports as the size of the polymer prevents it from forming an ordered crystalline structure. The electrospraying process caused pirfenidone to form an amorphous state inside the PLGA particles which commonly occurs drug particles prepared by this process. The extremely rapid evaporation of the solvent during the electrospraying process prevents drug molecules from forming an ordered crystalline structure. When the drug is dispersed inside the polymer an amorphous state is common. The relaxation of molecules in the amorphous state appeared as an endothermic peak near the end of the T_g for both the PLGA and the drug particles. This could be resolved
using modulated DSC as it allows for a slower heating rate and hence could separate the relaxation stage from the $T_g$ stage \cite{282}.

Coaxial electrospraying could be used to prolong the pirfenidone release profile. Coaxial electrospraying utilises two separate feeding capillary channels to produce a core–shell flow that can result in a core-shell morphology in the resulting particles where the inner core can be composed of different components to the shell. Immiscible solvents can be used to prepare a hydrophilic core to solubilise the pirfenidone and a more hydrophobic shell derived from a polymer to coat the core. Ho et al prepared core-shell nanoparticles of artemisinate using the same PLGA polymer used in this study for the shell. The inner core was composed of chitosan and artemisinate. The particles had a significantly reduced drug release as a result. This was due to the fact that the drug being in the core mean it would have diffuse slowly through small pores and as the polymer degrades \cite{255}.

Trivedi et al prepared pirfenidone-PLGA nanoparticles using a nanoprecipitation method for slow release formulation for corneal wound healing. The method was based on preparing a double emulsion in which one component contained the pirfenidone-PLGA solution another contained PVA solution. An emulsion was prepared from the two mixtures and ultrasonication ad evaporation was subsequently used to prepare the nanoparticles. A daily topical application of the pirfenidone nanoparticles into rat eyes was well tolerated. The formulation was also much more effective than pirfenidone solutions at corneal wound healing following an alkali burn \cite{221}. The pirfenidone encapsulated nanoparticles showed a much slower drug release to the control, more than 60% of the drug was released in less than 24 h \cite{283}.

The effect of DMSO on HTF cell viability (Figure 87) was evaluated to see what amount of DMSO can be used to solubilise the drug for the cell-based studies. Also, the study attempted to mix the ilomastat/ AZ 8955 as a solution with the IPC solution which then would start to form microparticle precipitates inside the IPC as the mixing process occurs. It was found that both ilomastat and AZ 8955 formed a solution at 100 mg/mL in 70% DMSO (30% EDTA solution). A mixture with small and homogenous particle size was achieved for both drugs. The presence of 7% DMSO triggered fibrillogenesis where the gel formed slowly even without EDTA dilution. It was observed that
this was due to the insolubility of EDTA in DMSO which formed insoluble crystals in the IPC in the presence of even small concentrations of DMSO.

It was noted that DMSO at concentrations above 1% caused a significant reduction in HTF cell viability when the incubation time exceeded 24 h. This could have been due to cellular toxicity where some of the mechanisms include cell cycle arrest, MMP inhibition, apoptosis and ion leakage from cells. The extent and type of DMSO toxicity has been shown to vary between different cell lines. For example, Singh, Mckenzie, and Ma showed that in an in-vitro assay none of the goat skin fibroblast cells survived beyond 3% DMSO. On the other hand, in peripheral blood mononuclear cells the cell viability was not affect by 10% DMSO but there was a reduction in cell proliferation, TNF-α, IFN-γ, and IL-2 production. In-vivo DMSO would be removed from the area by the flow of tissue fluid like (e.g. interstitial fluid) which means its cytotoxic effect is potentially much lower in-vivo. Rimso is an FDA approved formulation for interstitial cystitis and contains 50% DMSO.

A novel sulfamethazine-based anionic pH-responsive radiopaque embolic hydrogel has been developed for hepatocellular carcinoma therapy. The hydrogel is composed of pH-responsive an anionic block copolymer which exhibited a sol state at pH 8.5 which turns into a gel at physiological conditions (pH 7.4 and 37°C). Doxorubicin (anti-cancer drug) was loaded into the hydrogel through π-π interactions between the drug molecule and the phenyl rings in the hydrogel structure. This meant that the hydrogel depot exhibited sustained release of doxorubicin and inhibited the tumour growth in-vivo in rabbits.

In another study, a slow drug delivery system was prepared from a combination of 9-cis-retinyl esters loaded poly(D,L-lactide-co-glycolide) (PLGA) microparticles entrapped in an alginate hydrogel. The particles were prepared by the nonappreciation technique and then mixed with an alginate solution. Then calcium chloride solution was added to initiate the crosslinking of the gel. It resulted in a sustained release of the drug in-vivo in mice, both intravitreally and subcutaneously. The formulation showed no toxicity, scarring or a foreign body response to the depot implant.
AZ 8955 had a dose dependent effect on the cell viability of HTF cells (Figure 90) where the cell viability decreased to with the increase in drug concentration. This contrasted with ilomastat which did not show any signs of toxicity. At the maximum drug water solubility (140 µg/mL) the cell viability was 75% and 100% after a 72-h incubation for AZ 8955 and ilomastat. It can be due to AZ 8955 inhibiting a wider range of MMP enzymes than ilomastat (Table 14). As previously described, MMP enzymes are not only involved in wound healing but also in various processes vital for cell functions. MMP inhibitors have different effect on different cell lines as the function of the different MMP enzymes varies depending on the cell line [136]. The tissue is unlikely to be exposed to such concentrations of AZ 8955 due to the continuous flow of the aqueous humous and tissue fluid [240].

Ilomastat appeared to increase the cell viability (Figure 89) to 114% of the control at 50 µg/mL at a 72-h incubation. Bencsik et al showed that the inhibition of the MMP-2 enzyme by ilomastat had a cardioprotective effect in a rat model by significantly decreasing the infarct size compared. They also found that when evaluated at concentrations of 1.94 µg/mL ilomastat greatly increased the cell viability of cardiomyocytes in-vitro [289]. This was also shown by our study which showed that the cell viability of HTF cells increased in concentrations up to 50 µg/mL. At concentrations above 50 µg/mL the ilomastat effect on HTF cell viability started to disappear. Therefore, the cell protective effect might potentially be concentration dependent in HTF cells.

Pirfenidone showed no drop in the cell viability (Figure 88) when evaluated at concentrations up to 500 µg/mL. The cell viability started to decrease at higher concentrations and dropped to 1.5% at 5 mg/mL of pirfenidone. Shi et al also reported a significant decrease in cardiac fibroblast proliferation and viability at concentrations above 500 µg/mL [236]. The reduction in cell viability which became more apparent at higher drug concentrations was likely caused by the inhibition of proinflammatory cytokines such as TFG-β [290] and TNF-α [291]. The blockade of TGF-β1 has been shown to inhibit cell viability, migration and metastasis of mammary tumour in transgenic mice [290]. Wang, Reinach, and Lu reported that TNF-α promoted cell survival of corneal epithelial cells. TNF-α stimulated the activity of K+ channels and other cell signalling proteins involved in cell survival [291].
Clinically, pirfenidone has not shown toxicity even though it is administered at 500 mg doses orally [238]. Unlike the in-vitro assay, the local pirfenidone concentration during drug dissolution would likely be below toxicity levels. For orally administered solid dosage forms there is a continuous removal of the dissolved drug. The drug dissolution process happens simultaneously to the drug movement from the stomach across biological barriers into the blood stream [292].

In terms of MMP bioactivity, pirfenidone showed weak efficacy in terms of inhibiting collagen contraction (Figure 94). At 250 µg/mL, pirfenidone showed there was only a 12% reduction in collagen contraction at day 7 compared to the drug free control. This can be explained by the fact that although pirfenidone has been shown to inhibit MMP-9 and TIMP1, the IC50 values are much higher than those of ilomastat and AZ 8955. Pirfenidone anti-scarring effects are through other routes including the inhibition of TNF-α and TGF-β which were not possible to test using the contraction assay [236].

In contrast to pirfenidone, ilomastat significantly inhibited collagen contraction (Figure 96) and was more efficacious. At all the time points the concentration of ilomastat released was at a therapeutic level for all experimental conditions. The concentration of drug released was above the Ki values for the different MMP enzymes inhibited by ilomastat (Table 14). Ilomastat released from the IPC formulation on day 7 maintained the bioactivity profile (Figure 96) to the control (freshly prepared ilomastat at the same concentration). Ilomastat has been shown to inhibit the ECM production of collagen type I in HTF cells. This in turn may further aid in the ilomastat anti-scarring efficacy in-vivo [228].

AZ 8955 showed a significantly higher efficacy in collagen contraction (Figure 98) inhibition than ilomastat. At 50 µg/mL AZ 8955 resulted in an 82% reduction collagen contraction compared of the control. Ilomastat only reduced it by 49% at the same concentration. This was caused by several factors including the fact that the AZ 8955 is several orders of magnitude more efficacious at inhibiting MMP 2 than ilomastat. It inhibits a wider range of MMP enzymes which are also involved in the wound healing process (Table 14). MMP-2 plays a vital role in ECM remodelling and its expression in dermal fibroblasts has been shown to be prolonged after injury. MMP-2 is involved in
fibroblast migration and collagen remodelling in tissues \cite{133}. This can explain the cause of the strong collagen contraction inhibition. AZ 8955 is highly efficacious at inhibiting MMP-13 and MMP-1 which are involved in collagen degradation in the ECM during wound healing and their inhibition has been shown to greatly reduce scarring \cite{272}. The \textit{in-vivo} outcome might be different as ilomastat is a much more efficacious inhibitor of MMP-9 which is a major target in trichiasis \cite{142}. AZ 8955 released from the IPC formulation on day 7 showed the same bioactivity profile in terms of collagen contraction (Figure 98) and cell viability profile to the control (freshly prepared AZ 8955).
Chapter 5: The Potential of the IPC to Formulate Proteins and Cells
5. The Potential of the IPC to Formulate Proteins and Cells

5.1 Introduction

Gels have been considered for the formulation of protein-based medicines as slow release systems. Proteins are macromolecules which may limited mobility inside a hydrogel network. Gels that retain water can potentially stabilise and preserve the fragile tertiary structure of proteins \[293\]. Hydrogel based systems still suffer from issues such as mixing of the drug, injectability, biodegradability and the long term biocompatibility \[93\]. Mixing of a macromolecule such as a protein (e.g. antibody) in a hydrogel is not readily possible. Macromolecules do not readily mix, but for a protein to have any chance to mix with a gelling polymer such as collagen, it is necessary to premix the protein prior to gelling of the polymer.

Bevacizumab is a recombinant humanised monoclonal antibody used in the treatment of age-related macular degeneration (AMD) which is a condition characterised by angiogenesis at the macula in the retina of the eye. The antibody works by blocking angiogenesis by binding to vascular endothelial growth factor (VEGF) to inhibit its action. Bevacizumab has been shown to mediate healing after ocular surgery \[294\], \[295\] but the antibody is normally administered through intravitreal injections (IVT) \[296\] when used in the eye. IVT bevacizumab has an in vivo \(t_{1/2}\) of 6.7-10 days and therefore is administered monthly. Since AMD is a chronic condition, patients require long-term treatment so there is a need to reduce the frequency for injections. As well as being invasive and exceedingly stressful for patient, frequent intravitreal injections into the back of the eye may cause as side effects, including retinal detachment and ocular infection \[297\]. Therefore reducing the dosing frequency by preparing modified release formulations has attracted considerable research effort \[298\], \[299\].

The IPC fibrils that form yield meshes inside the gel that may sustain the release of bevacizumab. The IPC may be used to prepare a sustained release system of bevacizumab. The protein may be encapsulated within the fibrils. The high-water content within the IPC may help avoid protein aggregation. Also, the IPC offers the opportunity to administer an antibody-IPC using a small gauge needle (non-invasive) due to its in-situ gelling
properties. Bevacizumab is an IgG antibody which has a Mw of 150 kDa and a molecular size of approximately 14.5 nm × 8.5 nm × 4.0 nm [300]. Antibody release from the collagen mesh network in IPC might therefore be slower than that of pirfenidone (Mw= 388 Da) described in previous studies reported in this thesis.

Cell-based therapies are a promising tool to replace abnormal or injured tissues (e.g. neurones, cardiac muscles and bones) and to treat some diseases including cancer and neurodegenerative disorders [301]. Issues such as cell attachment and survival post-delivery and removal of cells by the immune system are plague the development many cell-based therapies. The physicochemical properties of the cellular microenvironment influence the state of differentiation of various cell types such as chondrocytes [302]. One approach to overcome these issues may involve encapsulating cells by entrapment within a hydrogel due to some physical similarity to the ECM (e.g. high water content, diffusibility of low molecular weight molecules) [93].

Hydrogels are thought to be useful as scaffolds for regenerative medicines because pores may be large enough accommodate living cells and to improve retention and viability of injected stem cells [302]. Hydrogels can may covalently incorporate cell membrane receptor ligands to stimulate adhesion, mobility and growth of cells within a hydrogel scaffold [93]. A 3D hydrogel-based system may allow for the preparation of more in-vivo biomimetic models for organ specific and disease specific studies. Most in-vitro studies have traditionally been carried out in a 2D environment where cells are grown on tissue culture plates. This environment has been seen to affect cell behaviour and signalling and therefore the subsequent effect of experimental drugs. In-vivo biomimetic models may allow a better understanding of many biological processes and diseased models to potentially reduce the use for animal studies that may not provide data of sufficient quality [179].

In-situ gelling polymers are good candidates to examine for cellular applications as such gels may allow for delivery using a small gauge needle and the gel can adapt to the area of the treatment. It is still difficult to provide the appropriate macro- and microenvironments for cell proliferation and tissue growth. Also long term biocompatibility of crosslinkers and the toxicity of
leachable, uncreated precursor reagents are some of the issues to overcome by an *in-situ* gelling polymer \(^{303}\).

The IPC hydrogel may be a good candidate for use in cellular applications as it is highly biocompatible, has tuneable mechanical properties and its physicochemical and biological cues may be developed to replicate the natural ECM. This IPC has been used to locally co-deliver two growth factors into the spinal cord (intrathecally) of Sprague–Dawley rats which had undergone a laminectomy. Tissue histology showed that the IPC was well integrated into the spinal cord tissues with no signs of scarring. The growth factor were locally released and there was significant improvement of the spinal cord injury compared to the control of growth factors only \(^{304}\).

### 5.1.1 Aims

One aim is to prepare and analyse a slow release *in-situ* gelling hydrogel formulation of bevacizumab using the IPC for subconjunctival delivery. HA will be examined as an excipient to further modify the antibody release. The second aim is to establish and characterise a method for preparing an injectable cell-IPC formulation using HTFs as model cells.

### 5.2 Materials and methods

#### 5.2.1 Materials

The following materials were purchased from commercial sources. Bevacizumab (HeteroDrugs, India); Pierce Silver Stain Kit (Thermo Scientific, US); NuPAGE 4–12% Bis-Tris gels; NuPAGE LDS sample buffer; (NuPAGE MOPS SDS running buffer (NP0001) and Novex sharp pre-stained protein standards (Life Technologies, UK); Instant Blue (Expedeon, UK); sodium hyaluronate (MW 1-1.8 MDa, Lifecore Biomedical, US); Live/Dead Cell Imaging Kit (Thermo Fisher Scientific, US); Celltiter-Glo 3D (Promega, US); 96-well plate (Thermo Fisher Scientific, US); Anti-Paxillin antibody (Abcam, UK), F-actin staining kit (Abcam, UK); Floromount-G (SouthernBiotech, US); Alexaflour-488 (Abcam, UK); human tenon fibroblasts were kindly donated by UCL Institute of Ophthalmology (London, UK). HCl, NaOH, phosphate buffered saline tablets, D-mannitol, EDTA disodium salt dihydrate, female luer...
coupler, dimethyl sulfoxide, human VEGF165 (V7259); glucose, bovine serum albumin, anti-human IgG; sodium hydrogen carbonate; goat serum; Triton-100x; 4% paraformaldehyde and tris-buffered saline were purchased from Sigma-Aldrich, UK.

5.2.2 Instrumentation

Sorvall Legend RT+ Centrifuge (Thermo Scientific, US); VirTis AdVantage freeze drier (SP Scientific, US); Spectramax M2 plate reader (Molecular Devices, US); EVOS FL microscope (Thermo Scientific, US); Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss AG, Germany)

5.2.3 Methods

5.2.3.1 Preparation and release of bevacizumab loaded IPC and IPC-HA

To prepare the IPC formulation, a purchased bevacizumab solution (~25mg/mL) was freeze-dried for 72h. The antibody pellet was then reconstituted at 40 mg/mL in in EDTA (35 mM, pH 7.0) solution. The bevacizumab solution (50 μL) was slowly mixed for 3 min with an IPC solution prepared earlier (450 μL) containing approximately 47 mg/mL collagen by using the syringe mixing method (section 2.2.3.4.1).

To prepare an IPC-HA formulation, PBS (810 μL) was used to dissolve HA (2.0 mg, 1.8 MDa) and a solution of bevacizumab (190 μL, 4.0 mg). The solution was lyophilised. The dried cake was reconstituted in EDTA (120 μL, 35 mM, pH 7.2) solution. The solution (40 μL) was mixed with the IPC (260 μL) solution for 3 min using the syringe mixing method (section 2.2.3.4.1). Air bubbles were removed by centrifuging at 4000 rpm for 3 min.

The mixture (100 μL) of Beva-IPC or Beva-IPC-HA was loaded into the ocular model (section in 4.2.3.2) using a positive displacement pipette. Samples of the released bevacizumab were collected after 4 h and then once daily afterwards. The cumulative drug release and the concentration of drug released were plotted against time.
5.2.3.2 Drug content determination

5.2.3.2.1 Chromatography

Bevacizumab concentration was analysed by using HPLC by constructing a standard curve for the antibody (Appendix Figure 124 and Figure 125). The injection volume was 50 µL. The flow rate was (1.0 mL/min) with a 100% PBS (pH 7.4) mobile phase. The column temperature was 30°C with a detection wavelength of 280 nm. The solutions were analysed with Agilent System fitted with Zorbax column 4 µm, 4.6×250 mm (Agilent, US). The run time was 12 min.

Drug loading into the IPC was determined by mixing solutions (5 µL) of Beva-IPC and Beva-IPC-HA with 50 µL of an EDTA solution (35 mM, pH 7.2). PBS (pH 7.4) solution was added (50 µL) was added to the solution which was then analysed for drug content using HPLC as described above.

5.2.3.2.2 Enzyme-Linked Immunosorbent Assay (ELISA)

The amount of active bevacizumab released was quantified using ELISA [299]. ELISA is a plate-based analytical technique which can detect and quantify peptides, proteins, antibodies and hormones. During the assay, an antigen is immobilised to a solid surface which then complexes with an antibody that is linked to an enzyme. Subsequently, detection is achieved by incubating the mixture with a substrate that can be catalysed by the enzyme to produce a measurable product (e.g. luminescence) [305].

Briefly, VEGF coated 96 well plates were prepared in advance by adding of VEGF solution (100 µL/well, 0.1 µg/mL) diluted in coating buffer (0.05 M bicarbonate buffer, pH 9.0) to an opaque high binding plate. The plate was incubated overnight (~18 h) at 4°C. The plates were washed (3×) with washing buffer (WB, 0.05% Tween 20 in PBS, pH 7.4). Blocking buffer (10% BSA in DI water, 200 µL) was pipetted into each well and was incubated for 1 h at an ambient temperature with shaking. The plate was washed (3×) with WB. Samples (100 µL/well) were then added to the wells and incubated for 2 h at an ambient temperature. The plate was again washed with WB (3×) followed by the addition of anti-antibody/PBS solution (0.2%). Next, the plate was incubated for 1 h at an ambient temperature and was washed (4×) with
WB. 1-Step Ultra TMB-ELISA was added to each well in the dark. HCl (100 μL, 1 M) was added to stop the reaction once a blue colour appeared in control samples. The plate was analysed to measure the luminescence intensity using a microplate reader. The bevacizumab concentrations were calculated using a standard curve of the antibody established using the same method described above.

5.2.3.3 Formulation Characterisation

5.2.3.3.1 Bevacizumab Stability in EDTA at 37°C

A stability study was carried out to study the effect of temperature (37°C) and the EDTA solution on bevacizumab stability. A solution of 35 mM EDTA containing 100 mM sucrose and 3.5% mannitol was prepared at pH 7.0. In 7 mL sealable vials, bevacizumab (20 μL, 25.0 mg/mL) was added to the EDTA solution (980 μL) to produce a 5 mg/mL antibody concentration. The vials were incubated at 37°C. Samples (100 μL) were taken at various time points and analysed with HPLC (using the method described earlier in section 5.2.3.2.1).

5.2.3.3.2 Bevacizumab Detection by Gel Electrophoresis

The presence of bevacizumab from the drug release studied was also monitored using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). This method separate proteins by size according the migration rate. It is a more sensitive qualitative test (not quantitative) at detecting the presence of proteins than HPLC \(^{306}\) and multiple samples can quickly be evaluated. The samples from the Bevacizumab-IPC formulations were qualitatively analysed with SDS-PAGE. To detect even lower protein concentrations a silver stain was carried out on an SDS-PAGE run gel. Silver stain is more sensitive than SDS-PAGE a alone \(^{306}\). SDS-PAGE in combination with a silver stain was carried out to analyse samples released from the IPC-HA formulation.

For the SDS-PAGE, samples were analysed using a Novex® Bis-Tris 4-12% precast gel in an electrophoresis tank. The samples (5 μL) were added to NuPAGE® LDS sample buffer (4x) (15 μL). The mixture (20 μL) was loaded into the PAGE gel. Novex sharp protein standard marker (3 μL) was also
loaded into the gel, molecular weight marker. The running buffer was NuPAGE® MOPS SDS (20×). The gels were resolved for 55 min at 200 V and 70 mA. The gel was stained with Coomassie blue for 1 h and washed with DI water for 1 h.

Pierce Silver Stain Kit was used for the sliver staining step. Samples from the IPC-HA formulations were run with SDS-PAGE first. Then, the gel was washed using ultrapure water for 5 min (2×). The gel was fixed with 30% ethanol:10% acetic acid solution (i.e. 6:3:1 water: ethanol: acetic acid) for 15 min (2×). The gel was then washed with 10% ethanol for 5 min (2×). Ultrapure water was used to wash the gel for 5 min (2×). The gel was incubated with a sensitiser working solution (50 μL sensitiser + 25 mL water) for 1 min and washed (2x) with ultrapure water. The silver stain solution (500 μL enhancer + 25 mL stain) was incubated with the gel for 30 min. The gel was washed with ultrapure water (1 min, 2×) followed by the addition of the developer solution (500 μL enhancer + 25 mL developer). The gel was incubated until darker protein bands appeared (2–15 min). A stop solution (5% acetic acid) was added to gel for 10 min and then removed followed by washing with ultrapure water. Controls of HA, bevacizumab only, collagen type I only and collagen type I and bevacizumab were also run simultaneously in both the SDS-PAGE and silver stain studies. The gels where scanned using scanner and the proteins were identified with respect to the molecular weight marker.

5.2.3.4 Preparation of IPC-Cell Formulation

5.2.3.4.1 EDTA Cytotoxicity in HTF Cells

HTF cells (passage 4-7) were seeded into 96 white well plates at a density of 10,000 cells/well. Cell suspension (75 μL) in complete DMEM was pipetted into wells. This seeding density (10,000 cells/well or 133,333 cells/mL) is commonly used in cytotoxicity studies [150], [307], [308]. The plate was incubated for 16 h in a humidified incubator at 37°C in 5% CO2. The media was removed and replaced with complete DMEM containing different concentrations of EDTA at pH 7.2. A control of HTF cells lacking any EDTA was also prepared under the same conditions. The plate was incubated for 24 h under the same
conditions. The cell viability was analysed using CellTiter-Glo® 3D as described in section 2.2.3.3.1.2

5.2.3.4.2 IPC-Cell Preparation Method

To establish the formulation method, several parameters were optimised including ratio of cell suspension to IPC and mixing times. The amount of media added to the cell-IPC solution was also pre-optimised to account for the fact that the EDTA would be continuously removed (by diffusion and dilution) from the in-vivo area and would therefore not be exposed to the HTF cells over a prolonged period. In this in-vitro assay the amount of EDTA cells are exposed to does not change with time.

HTF cells (passage 4-7) were pelleted using centrifuging at 180 g for 7 min, at 21°C. The cell pellet was resuspended in complete DMEM in 15 mM EDTA at 10 Million cells/mL. The cell suspension (250 µL) was mixed (using the syringe mixing method) with the IPC (250 L) containing 30 mg/mL of collagen. The mixing time was 5 min followed by centrifuging at 180 g at 4°C for 10 min to remove air bubbles created by the mixing process.

5.2.3.4.3 Cell Viability

To test the cell viability and realignment of HTF cells embedded in the IPC gel the preparation prepared above was analysed. For the cell viability study, the IPC-cell solution (25 µL) was pipetted into 96 well plates using a positive displacement pipette. Complete DMEM media was then added (225 µL) to the well to induce gelling (fibrillogenesis). The plate was incubated for 1, 3 and 7 days at 37°C and 5% CO₂ in a humidified incubator. The positive control was HTF. At each time point, the cell viability was analysed using CellTiter-Glo® 3D as previously described in section 2.2.3.3.1.2.

A control of HTF cells embedded in 15 mg/mL pepsin soluble (PS) collagen gel was prepared by neutralisation as previously described (section 4.2.3.6.3). The PS collagen (6 mg/mL) was concentrated to 10 mg/mL by using the salt precipitation method previously described followed by acid dialysis for 48 h (section in 3.2.3.1). The HTF embedded collagen gel was then prepared using the neutralisation method (section 4.2.3.6.3). The neutralised collagen-
cell solution (25 µL) was pipetted into 96 well plate and then left to gel (20 min at 37°C). Complete media (250 µL) was added to wells after gelling.

A cell viability study was also conducted to test whether cells can be mixed with the IPC solution prior to application or whether mixing immediately before use was required. Briefly, the prepared cell-IPC solution was left at ambient temperature in an Eppendorf vial for 0, 1 and 3 h receptively before being pipetted (25 µL) into 96 white well plates. After each time point, complete media (250 µL) was added to the wells. The control was PS collagen embedded cells prepared as previously described where media was added immediately after gelling was complete. The plate was incubated for 24 h in a humidified incubator as described earlier. The cell viability was measured using CellTiter-Glo® 3D as previously outlined (section 2.2.3.3.1.2).

5.3.4.4 Cell Realignment

A contraction assay was utilised to monitor whether HTF cells embedded in IPC result in gel contraction, i.e. become realigned. The IPC-cell solution was prepare as described above. IPC-cell solution (150 µL) was pipetted into glass bottom (MatTek) dishes at a 1 Million cells/mL seeding density. Complete DMEM (2 mL) was added to the well and the dishes were incubated at 37°C and 5% CO₂ in a humidified incubator. A positive control of HTF cells (at 1 Million cells/mL) embedded in 4 mg/mL acid soluble bovine (AS) collagen gels was prepared using the neutralisation method. HTF cultures in 4 mg/mL AS collagen showed the contraction levels to the 2 mg/mL rat tail collagen in previous experiment.

To control of the effect of telopeptides, HTF cells embedded in AS collagen containing 15 mg/mL collagen (concentrated using salt precipitation) were also prepared (150 µL) by the neutralisation method. To control for the presence of EDTA and the preparation process, HTF cultures (at 1 Million cells/mL) were embedded in 15 mg/mL PS collagen gels (150 µL) using the neutralisation method. The AS- and PS-cell solutions were left to gel for 15 and 20 min respectively before the addition of media (2 mL).

Gel contraction was monitored daily for 7 days using image photography. The extent of contraction was then measured as previously
described (section 4.2.3.6.3). Collagen cultures from the contraction assay experiment were stained for live and dead cells at day 1 and 7. The live dead staining mixture was prepared by mixing component A and B. Then, 1 mL of the media was removed and replaced with 1 mL of the staining reagent. The dishes were incubated for 15 min at 37°C and 5% CO₂ in humidified incubator. The gels were then imaged using a fluorescent light microscope as previously described (in section 4.2.3.6.5)

5.2.3.4.4.1 Immunofluorescent Staining

To ensure cells embedded in the IPC gel were attached to the gel and can therefore function, the cell cytoskeleton was imaged. Samples of HTF cells embedded in collagen gels (AS at 4 mg/mL and IPC) prepared in the contraction assay experiment were utilised for staining after 24 h of incubation.

The media was removed, and the gel was transferred into a 24 well plate. Each gel was washed with PBS (500 µL, pH 7.4). The gel was then incubated with 4% paraformaldehyde (PFA) (500 µL) for 10 min at ambient temperature. The PFA removed after 10 min and the gel was washed (3x) with cold PBS (500 µL) (refrigerated). To block any nonspecific binding a solution (500 µL) of 1x tris-buffered saline containing 0.3% Triton-X100 (TBS-T) in combination with 3% goat serum was added to the gel. The gel was then left for 30 min at ambient temperature.

The anti-paxillin primary antibody (rabbit recombinant monoclonal Paxillin antibody [Y113]) was diluted with 1% goat serum in TBS-T (1:100) and incubated (500 µL) with the gel at 4°C overnight. The gel was washed (3x) with tris-buffered saline (1x, TBS). A solution of Alexaflour-488 was diluted (1:500) in 1% goat serum in TBS-T was added to a solution diluted F-actin (1:500) at 1:1 ratio. A solution (500 µL) of the mixture was then added to the gel and left to incubates in the dark for 2 h. The solution was removed the gel was washed with (3x) with TBS (500 µL). A solution of DAPI (5 mg/mL) diluted 1:5000 in TBS was added (500 µL) to the well and left for 10 min at ambient temperature in the dark. The gel was washed (3x) with TBS. The gel was mounted on a Superfrost-plus slide and a drop of Floromount-G was added before covering with a coverslip. The edges were sealed with a clear nail
varnish. These were then left to dry overnight in the dark at ambient temperature. The slides were analysed with a laser scanning confocal microscope using a 63×magnification lens.

5.2.3.5 Statistical Analysis

The data sampling was carried out and statistically analysed as described in section 2.2.3.5. In this chapter, the samples were prepared in triplicates and the experiments were repeated on at least 3 separate occasions. Error bars represent the SE as the number was at least n=3. ORM-ANOVA was used to analyse the cell viability in IPC. The rest of the data was analysed using F-ANOVA. A minimum significance level of p< 0.05 was used for all statistical tests [160].

5.3 Results

5.3.1 Bevacizumab Hydrogel Formulation and Release

The bevacizumab-IPC (Beva-IPC) formulation release was tested using the flow rig ocular models to mimic intraocular release (as described in section 4.2.3.2). The formulation was situated in a small chamber (200 µL) and exposed to a continuous flow of media. The rate of flow was ~2 µL/min same as that of the aqueous humour.

Bevacizumab was formulated with the IPC and the loading was determined to be ~4 mg/mL (using HPLC). For a 100 µL Beva-IPC depot the drug release (Figure 102) occurred over a 7-day period. Time had a significant effect (p< 0.01, within subject F-ANOVA) on the drug release profile. The burst release at 4h was 5.8% which also represented the maximum concentration (Cmax) which was 61.0 µg/mL. The cumulative release increased to 30.0% after 24 h. Only 64% of the drug was released by 7 days after which no drug was detected. The release profile of a solution of bevacizumab only was not studied as it is was pre-established in Chapter 4 that the release of a drug solution loaded into the release chamber was completed within 2 days (section 4.3.4.1).
When SDS-PAGE was used for protein detection (Figure 103, next page) bands corresponding to the collagen molecular weight appeared in both the collagen control and in the release samples (although faint). Bands consistent with intact bevacizumab (~150 kDa) were detected in the control and the release samples up to day 9 (faint bands) but not on day 10. The bands corresponding to the antibody fab fragments at 50 kDa were not detectible during the study when SDS-PAGE was used.

**Figure 102.** The in-vitro release profile of bevacizumab from the IPC gel formulation at 37°C. The cumulative release reached 70%. The values represent the mean ± SE and at n=3.
Hyaluronic acid (HA) is a biocompatible and biodegradable polymer which swells and forms a viscoelastic solution in water. The incorporation of HA into the IPC might swell inside the collagen meshes and to reduce the size of the meshes. HA (~1.8 MDa) was formulated with the IPC, (IPC-HA) at ~4 mg/mL of bevacizumab loading. The release from 100 µL depot (Figure 104, next page) occurred slowly over 30 days at which the study was terminated.

ELISA was used to determine drug content as no drug was detected after 16 days of release using HPLC. ELISA represented the amount of antibody present that could still bind to VEGF, showed that bevacizumab still released for another 13 days. The bevacizumab release was lower at all time points when ELISA was used for determining drug content, indicating evidence of protein binding.

Figure 103. Novex Bis-Tris 4–12% gel loaded with samples collected from release study of the IPC (Lanes 1-15). Lane M: molecular weight standard; Lanes C1-C3 are Controls: collagen (C1) bevacizumab & collagen (C2) bevacizumab (C3). Lanes 1-15: are released samples from Beva-IPC. Lanes 1-3: day 1,4-6: day 3, 7-9: day 6, 10-12: day 9 and 13-15: day 10.
The $C_{\text{max}}$ was reached at 24 h as determined by both detection methods and were 37.0 and 47.0 µg/mL for ELISA and HPLC respectively. The release became constant after day 5 and remained over 200.0 ng/mL. The proportion of bevacizumab detected by ELISA compared to that detected by HPLC was approximately 83.4%. This indicated that there was about 16.6% loss in binding affinity, presumably due to mis-folding, denaturation, aggregation or degradation to smaller fragments that cannot bind to VEGF.

In the silver stained SDS-PAGE gels, bands at 150 kDa appeared with some traces of bands at 50 kDa (Figure 105) appeared at all of the time point of the release study, up to day 30. These bands were consistent with the molecular weight to intact bevacizumab (150 kDa) and fab fragments (50 kDa). Other bands were consistent with molecular weight of collagen at around 300 kDa.

Figure 104. The in-vitro release profile of bevacizumab from IPC-HA formulation at 37°C using ELISA and HPLC as analytical techniques. The values represent the mean ± SE and at n=3. p values< 0.001 when the concentration profiles and the cumulative release profiles of each technique were compared using F-ANOVA. * = Statistically significant (p< 0.05).
When the Beva-IPC formulation (without HA) was compared to the release profile of pirfenidone-IPC (Figure 106) it could be seen that bevacizumab release was much slower. For example, at 24 the cumulative release was 96 and 35% for Pirfenidone-IPC and Beva-IPC respectively. Bevacizumab was released at an even slower rate in the IPC-HA gel where only 20.0% was release at 24 h.

**Figure 105.** Silver stain of a Novex Bis-Tris 4–12% gel loaded with bevacizumab released from IPC-HA. Lane M: molecular weight standard and Lanes 1-21: released bevacizumab at different time points. Lanes 1-3: day 1, 4-6: day 3, 7-9: day 6, 10-12: day 9, 13-15: day 12, 16-18: day 15, 19-21: day 18, 22-24: day 21, and 25-27: day 27 and 28-30: day 30.

When the Beva-IPC formulation (without HA) was compared to the release profile of pirfenidone-IPC (Figure 106) it could be seen that bevacizumab release was much slower. For example, at 24 the cumulative release was 96 and 35% for Pirfenidone-IPC and Beva-IPC respectively. Bevacizumab was released at an even slower rate in the IPC-HA gel where only 20.0% was release at 24 h.

**Figure 106.** The in-vitro release of bevacizumab and pirfenidone from the IPC formulation compared to bevacizumab released from the IPC-HA formulation at 37°C. Beva= bevacizumab. The values represent the mean SE and at n=3, p values < 0.001 when the different release profiles were compared using F-ANOVA. * = Statistically significant (p< 0.05).
5.3.2 Bevacizumab Thermal and EDTA Stability

The experiment showed that there appeared to be a decrease of 30% in the bevacizumab concentration with time (p< 0.001) by day 10 (Figure 107). It was curious that the bevacizumab loss in the presence of EDTA and mannitol, which is not a reducing sugar, was similar to the amount of unreleased bevacizumab from the IPC and IPC-HA formulations. The cumulative amount of bevacizumab released was 68.0 and ~64.0% from the IPC and IPC-HA formulations suggesting there was approximately 30-40% of the bevacizumab still in each formulation. It is possible that the formulation process or the excipients (mannitol, EDTA, sucrose and collagen) as well antibody losses during release and analysis might have resulted in decrease in total bevacizumab detected by HPLC.

Figure 107. The change in the total bevacizumab with time in EDTA, mannitol and sucrose solution stored at 37°C. The solution contained 5 mg/mL bevacizumab in 35 mM EDTA, 100 mM sucrose and 3.5% mannitol, at pH 7.2. The values represent the mean ± SE and at n=3.
5.3.3 IPC-Cell Formulation

Establishing the effect of cell behaviour inside an IPC gel and a formulation method is important. This study aims to establish an IPC-cell preparation method using HTF cells as model cells. HTFs are attachment dependent cells that interact with the ECM. HTFs are primary cells which are more sensitive to external stimuli than secondary (immortalised) cell lines and this thesis is mainly focused on ocular applications.\(^{[309]}\)

Certain cell therapies would be expected to require cell realignment inside the hydrogel system such as those in peripheral nerve repair.\(^{[310]}\) Collagen based contraction assays were used to study cell realignment \textit{in-vitro}\(^{[149]}\). HTFs are commonly used in contraction assays\(^{[133],[141]}\) and were conducted for 7 days to investigate the effect of the IPC on cell realignment.

The cell density used was 5 Million cells/mL, similar to that in \textit{in-vivo} studies for tissue engineering.\(^{[311]}\) Alofisel\(^{®}\) which is injected into the fistula tract tissue is used at 5 Million cells/mL.\(^{[96]}\) When cells attach to surfaces the actin protein inside the cell polymerises to form F-actin as part of the cytoskeleton and is an indication of cell attachment. Paxillin is another cytoskeletal protein involved at sites of cell adhesion to the ECM (focal adhesion) and is also utilised during cell attachment. Both proteins are involved in cell migration which important in processes like re-alignment of the ECM.\(^{[23]}\) Therefore, a red fluorescent phalloidin conjugate will be used to selectively bind to F-actin.\(^{[312]}\) An anti-paxillin antibody in combination with a secondary antibody (Alexaflour-488) will be used to stain the focal adhesion points.\(^{[313]}\) Also, 4′,6-diamidino-2-phenylindole (DAPI) will also be used to stain the nucleus of the cells.\(^{[314]}\)

It was found that a 1:1 ratio of IPC:cell suspension was ideal in-terms of mixing time and the homogeneous distribution of cells inside the IPC. The mixing time could be decreased by lowering the starting concentration (from 47.0 to 30.0 mg/mL). The addition of media to induce fibrillogenesis impaired the gel integrity when the final collagen concentration of IPC was below 15.0 mg/mL. For example, at 10.0 mg/mL (Figure 108) the IPC gel appeared weaker and split in certain areas and did not maintain its shape. The gel of HTF cells embedded in 15.0 mg/mL collagen in IPC was complete and
maintained its shape. Therefore, when used in in-vitro studies the collagen concentration in the IPC should ideally be above 15.0 mg/mL to achieve a fully formed stable cell embedded gel.

![Image of collagen concentration comparison (10 mg/L vs 15 mg/mL)](image)

**Figure 108.** The lack of complete gel formation at 10 mg/mL collagen IPC vs 15 mg/mL. 5 Million cells/mL of HTF cells embedded in IPC.

### 5.3.3.1 Effect of EDTA on Cell Viability

The cell viability of HTF cells in EDTA (**Figure 109**) was monitored to measure the effect on cell viability in different concentrations. The cell viability remained high (above 80.0%) in up to 1.88 mM of EDTA. The cell viability of HTF cells dropped significantly at higher concentrations after a 24 h incubation. For example, the cell viability was 15.0% at 15 mM EDTA. The EDTA concentration had a significant effect (p< 0.001, within subject F-ANOVA) on the cell viability.

![Graph showing cell viability vs EDTA concentration](image)

**Figure 109.** The change in HTF cell viability after a 24 h incubation in different concentrations of EDTA (pH 7.4) at 37°C and 5% CO₂. The values represent the mean ± SE and at n=3.
5.3.3.2 Cell Viability in IPC

The cell viability of HTF cells embedded in IPC gels (Figure 110) remained high (above 90.0%) at the different time points (day 1, 3 and 7). The final concentration of EDTA in the well was maintained at 1.50 mM to allow for lack of EDTA removal over 24 h that usually occurs by diffusion and dilution in vivo. The incubation time did not have a significant effect (p= 0.861) on cell viability. The formulation process (mixing and centrifuging) and exposure to EDTA and the IPC did not affect the cell viability of HTF cells.

The effect of incubating HTF cells in the IPC solution for different amounts of time (Figure 111) before the solutions were set into gels by culture addition of media was then examined. Cell viability decreased with time (p < 0.001). The cell viability was above 90.0% when the mixture was immediately set into a gel after preparation by the addition of complete DMEM. When the gel setting was delayed, the cell viability dramatically decreased with time to approximately 30.0% when cells where left in the IPC solution for 3 h.

Figure 110. The cell viability of HTF cells embedded in IPC gels relative to PS gels after different incubation times (1, 3 and 7 days) at 37°C and 5% CO2. PS control is neutralised pepsin soluble collagen. Both gels contained 15 mg/mL collagen type I. The values represent the mean ± SE and at n=3, p= 0.861 when the cell viability data of different incubation times were analysed using ORM-ANOVA. N/S= Not statistically significant (p> 0.05).
5.3.3.3 Cell Realignment

The effect of the IPC and collagen concentration on collagen contraction (Figure 112) was evaluated to analyse the effect of IPC on collagen contraction. No contraction was seen after 7 days of contraction monitoring of 5 Million HTF cells/mL of gel. The lack of contraction was seen in both PS and AS collagen gels (prepared by neutralisation) containing the same concentration of collagen (15.0 mg/mL) as the IPC. AS gel containing 4 mg/mL collagen showed a significant contraction on day 1 (64.0%) and remained relatively constant over the remaining time up to 7 days. Collagen concentration had a significant effect (p< 0.001) on gel contraction in AS collagen.

**Figure 111.** The change in cell viability of HTF cells incubated with an IPC solution at an ambient temperature for different times (0, 1 and 3 h) before gelling and a further 24 h incubation was conducted. After respective incubation times, the IPC-cell solution was gelled (in complete DMEM) and incubated at 37°C and 5% CO₂ for 24 h before the cell viability was evaluated. The values represent the mean ± SE and at n=3, p < 0.001 when the different time profiles were compared using ORM-ANOVA. * = Statistically significant (p< 0.05).
When cells embedded in IPC gels were stained using a Live-Dead staining kit (Figure 113) there was no reduction in cell viability. At 24 h the cells embedded with 15 mg/mL of collagen (PS and IPC) were viable but showed a rounder and less elongated morphology. At day 7, the cells appeared more elongated and remained highly viable. Cells embedded in 4.0 mg/mL AS collagen remained highly viable. It was not possible to distinguish more details (both on day 1 and 7) due to the high proximity of the cells to each other caused by the significant gel contraction.

**Figure 112.** The 7-day gel contraction profile of HTF cells embedded in different collagen gel preparations (15 mg/mL collagen) compared to the control. AS= acid soluble collagen (Control, 4 mg/mL collagen), IPC= In-situ polymerisable collagen and PS= pepsin soluble collagen. At 15 mg/mL of collagen the gel contraction was 0% at all time points for all the different collagen gel preparations. The values represent the mean ± SE and at n=3. p values< 0.001 when the different profiles were compared to the control using F-ANOVA. * = Statistically significant (p< 0.05).
**Figure 113.** The change in morphology of HTF cells embedded in 15 mg/mL collagen gel over time in IPC gel (A and B), PS collagen gel (B and C) and 4 mg/mL AS collagen gel (E and F). The HTF cells appear more elongated on day 7. Live cells (Calcein AM, green) and dead cells (nuclear dye, red) stained with a Live-Dead Staining kit.
5.3.3.4 Cell Cytoskeleton and Morphology

To assess how well the cells were attached to the collagen inside the IPC compared to the control (4 mg/mL AS collagen gel) both cytoskeleton and the focal adhesion points where stained after 24 h of HTF gel construct preparation (Figure 114 and Figure 115). Polymerised actin (F-actin) and paxillin were present in both IPC and AS (4 mg/mL) collagen cultures. The sites of F-actin and paxillin were overlapped in certain areas. These observations indicated that the HTF cells were fully attached to the collagen gels. However, cells in AS collagen (4 mg/mL) were more elongated and fibroblast-like. There was a higher intensity of F-actin and it was also more defined in AS embedded HTF cells. This was likely due to the cell re-alinement (collagen contraction) resulting in more polymerisation of F-actin and the cytoskeleton to be under more tension.

Figure 114. HTF cells cultured in IPC for 24 h stained for F-actin and Paxillin. The nucleus stained with DAPI (blue), the F-actin stained with phalloidin conjugate (red) and focal adhesions with anti-paxillin primary antibody and the secondary Alexaflour-488 antibody (green). B) and D) are F-actin stain and A) and C) overlay of F-actin and paxillin staining.
5.3.4 Summary of Results

The IPC was successfully used to formulate an *in-situ* gelling antibody formulation with bevacizumab. Beva-IPC showed a 7-day release profile where the total release was 68.0%. When HA (~1.8 MDa) was incorporated into the IPC, the release was extended to 30 days. However, both formulations showed a loss of total bevacizumab. The bevacizumab released from the IPC-HA formulation displayed over 80% of its VEGF binding capacity compared to total antibody released.

A preparation method for a cell IPC formulation was established using HTFs as model cells. The HTF embedded IPC gel showed excellent cell viability 7 day after incubation but failed to show any signs of gel contraction at 15 mg/mL collagen. Gel contraction in cell embedded AS collagen gels was concentration depended where the 4 mg/mL collagen showed significant contraction but failed to contract completely at 15 mg/mL collagen. When HTF

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*Figure 115.* HTF cells cultured in AS collagen gels (4 mg/mL) for 24 h and stained for F-actin and paxillin. The nucleus stained with DAPI (blue), the F-actin stained with phalloidin conjugate (red) and focal adhesions with anti-paxillin primary antibody and the secondary Alexaflour-488 antibody (green). **A)** and **B**) are F-actin stain and **C)** and **D)** overlay of F-actin and paxillin staining.
cells embedded in IPC gel were stained for paxillin and F-actin the cells where shown to have attached fully using their focal adhesion points.

5.4 Discussion

Bevacizumab release from the IPC (Figure 102) was slow and occurred over 7 days. This was likely due to the size of the antibody (14.5 \times 8.5 \times 4 \text{ nm}) \textsuperscript{300} which likely slowed its diffusion through the collagen mesh (\leq 300 \text{ nm}) in the IPC. The release was much slower than that of pirfenidone (Figure 106) which is a much smaller molecule. The burst release was low (5.9\%) and overall bevacizumab release was only 67\% and no further antibody was detected by HPLC after 7 days. This was likely due to loss of antibody as shown by the stability study (Figure 107). There was a 30.0\% loss of antibody when the bevacizumab was stored in a solution of EDTA at 37^\circ \text{C} for 10 days. This indicted that bevacizumab may have misfolded, aggregated or denatured. Chemical degradation of bevacizumab was also possible. The products could not be detected by the HPLC at the same retention time. The degradation was most likely caused by the thermal stress which can result in thermal induced oxidation and hydrolysis \textsuperscript{315}. EDTA does not affect the protein structure at the concentrations used in this study. It has even been used to prevent ion induced oxidation of antibodies \textsuperscript{316}.

Experimentally, a PNIPAAM collapsable gel has been used to formulate antibodies for intravitreal delivery. The high burst release of the antibody (human IgG) was higher in these studies (> 40.0\%). The long term clearance and safety of the the polymer in the eye remained issues to solve \textsuperscript{317}.

The inclusion of HA (2 mg/mL) into the IPC system resulted in an even slower antibody release profile (Figure 104) which was still releasing on day 30 when the study was terminated. According to the ELISA assay the release was relatively constant after day 5 and did not drop below 200 ng/mL throughout the study. As mentioned previously HA swells in the presence of water potentially resulting in the reduction in mesh size of the IPC to reduce bevacizumab release from the IPC. ELISA is a functional assay in protein drug delivery which is important to ensure that the protein has not detriorated during dosage form fabrication, storage or release period.
There was evidence that bevacizumab displayed some loss of binding when release samples from the IPC-HA formulation were analysed by ELISA. HPLC analysis measures total protein and up to day 16 indicated that 64.0% of the antibody had been released (Figure 104). HPLC analysis could not detect the released antibody after 16 days, but ELISA indicated that antibody was still being released until day 30. For both ELISA and HPLC the drug release after day 5 remained relatively constant. ELISA which provides a measurement of the amount of antibody capable of binding to VEGF indicated that there was a loss in antibody throughout the release period.

The binding of bevacizumab to VEGF (in ELISA) showed that over 80.0% of total released protein was active. Most of the released protein maintained its anti-VEGF binding throughout the release period. Intact aggregated and fragmented antibody can also bind to VEGF and the ELISA release values may have also include aggregated and possibly fragmented bevacizumab, since silver stained SDS-PAGE gels confirmed the presence of Fab fragment at 50 kDa. The loss of total antibody and the decrease in antibody binding in both formulations (Beva-IPC and Beva-IPC-HA) can be caused by several factors. Protein precipitation, aggregation or misfolding might have occurred during the formulation and release process. Likewise, degradation of the protein mainchain is also possible. The hinge region of an antibody is particularly susceptible to fragmentation.

There are several steps in the processes used to make the bevacizumab IPC formulations that could result in loss of antibody and loss of antibody function. In addition to the apparent loss of antibody when incubated with EDTA/mannitol/sucrose, the mixing of IPC with bevacizumab solution could have resulted in loss of antibody. Also, some antibody may absorb to the tubing and vials throughout the release period or to the HPLC column during analysis. Lyophilisation has been also shown to result in loss of antibody binding. It has been shown that the inclusion of potassium HA with bevacizumab prior to lyophilisation greatly preserved its binding affinity. Sodium HA can therefore be replaced with potassium HA when formulating Beva-IPC-HA in the future.

Several excipients have been used to increase the solution stability of antibodies including and sucrose which is already present in the IPC
solution. Other excipients which have been to increase the stability of proteins are amino acids like glutamate \textsuperscript{315}, histidine \textsuperscript{315}, arginine \textsuperscript{319} and lysine \textsuperscript{320}. Arginine and glutamic acid reduce antibody aggregation by increasing the solubility of the protein \textsuperscript{319}, \textsuperscript{320}. These amino acids may be incorporated into IPC formulations to increase the stability of bevacizumab.

The concentration of protein with anti-VEGF activity was above 200.0 ng/mL throughout the study (Figure 104). Takayuki et al intravitreally injected a bevacizumab solution (Avastin) in patients to treat proliferative diabetic retinopathy. They established that 83.0 ng of intact bevacizumab (MW= 150 kDa) was required to block VEGF in the vitreous \textsuperscript{321}. Although, the levels of VEGF are raised in both diabetic retinopathy and AMD the amount of bevacizumab required to block VEGF might be different due to different disease pathologies \textsuperscript{322}. This indicates that the IPC-HA formulation may potentially maintain therapeutic anti-VEGF levels intravitreally for a prolonged period.

Recently, Zhang et al prepared bevacizumab PLGA encapsulated nanoparticles which showed a slow release of 20 days. However, 40.0% of the drug was released at 2 h and approximately 70.0% was released by day 5. The nanoparticles were well tolerated and histology staining showed no signs of cytotoxicity \textsuperscript{323}. Thackaberry et al conducted a toxicology study on PLGA by injecting (IVT) PLGA microspheres and implanting a PLGA rod shaped implant (3.0 mg rod) into rabbit and monkey eyes. The study was conducted for 39 weeks and showed that rods were tolerated and showed no immune response. The microspheres showed a time and dose dependent foreign body response \textsuperscript{324}. The PLGA bevacizumab nanoparticle formulation may potentially have long term effects which were not detected due to the short length of the in-vivo study.

Fahmy-Garcia et al recently reported very promising in-vivo results for their in-situ gelling hydrogels loaded with collagen-like peptide (RCP) microspheres as a slow-release system to induce ectopic bone formation rats \textsuperscript{325}. Dry microspheres were first prepared by emulsification and subsequently crosslinked using hexamethylene diisocyanide by dehydrothermal crosslinking. The bone morphogenetic protein 2 (BMP-2) was then adsorbed onto the particle surface due to microsphere swelling which occurred during
the re-wetting process of the particles \(^{[326]}\). The microspheres were dispersed in a HA-based (thermosensitive) or alginate-based (shear-stress responsive) hydrogel which form gels upon injection. The mixture was subcutaneously injected into rats and a gel forms depending on physical stimuli. The adsorption efficient was only 16.0 µg of BMP-2 per 1 mL particles. This can be an issue as the equivalent human dose would potentially be larger for any therapeutics efficacy to occur \(^{[269]}\). Also, the particles size being approximately 75 µm in diameter means that 19G needles are required for injections \(^{[327]}\). Much smaller needles are usually needed for intraocular injection (e.g. 25-29 gauge) \(^{[326]}\).

When HTF cells were incubated in different concentrations of EDTA for 24 h there was a dose dependent effect on cell viability (Figure 111). The EDTA toxicity is related to its ion chelating activities where it can chelate divalent ions such as Ca\(^{2+}\) and Mg\(^{2+}\) ions which are vital for cell function \(^{[328]}\). However, EDTA is clinically administered intravenously at 10.0% to treat lead and mercury poisoning \(^{[111]}\). EDTA is also clinically used in certain wound dressings to manage wound healing (MMP inhibitor) and infections \(^{[328]}\). This indicates that the effect EDTA is time dependent and therefore the in-vivo effects of IPC-cell formulation will likely not be toxic as the EDTA would constantly be removed and diluted at the implant site.

The IPC (in 35.0 mM EDTA) successfully passed stage II trials with any reactions when injected subcutaneously for tissue augmentation\(^{[109]}\). The result of the study means that in in-vitro studies that use IPC-cell preparation the total concentration of EDTA in the solution needs to be kept below 1.87 mM to account for the lack of removal of EDTA from the experimental well.

A formulation method was successfully established, and the IPC-cell formulation showed a very high (> 90.0%) cell viability even after 7 days of incubation (Figure 110). Incubation of HTF cells in the IPC solution showed a significant reduction in HTF cell viability (~40.0 %) even after 1 h (Figure 111). When establishing the preparation method during preliminary studies, the EDTA content in the IPC-cell formulation was diluted to 3.75 mM for 1h. Then the EDTA was diluted further to 1.5 mM for the remaining 23 h with no effect on cell viability (> 90.0%). The cells may therefore need to be mixed with the IPC solution just before delivery.
Espona-Noguera et al also prepared an injectable alginate-based hydrogel for cell therapy. They used rat insulinoma (INS1-E) cells as model cells for insulin secretion for diabetes mellitus. Their study analysed the hydrogel preparation in-vitro with good cells viability and insulin secretion properties. They added sodium phosphate to slow the gelling of alginate and allowed it to be injectable. The gelling started during the syringe mixing process alginate with the cells which can be an issue\textsuperscript{[329]}. The ionic crosslinks (by Ca\textsuperscript{2+} ions) which hold the hydrogel together can cause gel break down in an unpredictable manner. The alginate polymer has purity issues can cause major reactions as the polymer is derived from seaweed\textsuperscript{[330]}.

The gel contraction of HTF embedded collagen was concentration dependent AS collagen (Figure 112). After 7 days AS collagen showed a significant contraction at 4 mg/mL collagen but no contraction at 15 mg/mL. Although only tested at a concentration of up 2 mg/mL, telocollagen (acid soluble) has shown a concentration dependent level of contraction\textsuperscript{[311]}. PS and IPC collagen gels which contained 15 mg/mL of atelocollagen (pepsin soluble) showed no contraction after 7 days. In previous experiments PS collagen (6 mg/mL) showed no contraction after 24h. The lack of telopeptides has been shown to affect collagen contraction\textsuperscript{[24]}.

When HTF cells where stained for focal adhesion points (paxillin) and the cytoskeleton (F-actin). The HTF cells embedded in the IPC were round after 24 h compared to the cells embedded in AS collagen (4 mg/mL) (Figure 113). Cells in both gels showed attachment to the gel and the formation of F-actin. However, cells in AS collagen had more F-actin with higher level of polymerisation\textsuperscript{[23]}. This was probably due to the cell realignment inside the gel which allows the cell to pull out of the gel matrix. On day 7 the IPC embedded cells showed a more elongated morphology (Figure 113). This is was likely due to the slowdown in cell migration by the higher collagen concentration. The 3D environment of the cells is known to affect the way that cells spread and attach\textsuperscript{[301]}.

In a recent study conducted by Gau et al they successfully prepared an injectable self-healing conductive hydrogel based on N-carboxyethyl chitosan and dextran-graft-aniline oligomers which was biodegradable. It was used as a cell delivery carrier for myoblast cell therapy to enhance skeletal tissue repair.
and showed very promising results when injected in a rat model of volumetric muscle loss injury of the skeletal muscle. However, the long-term biocompatibility and toxicity was not studied. This preparation is not suitable for applications where cells release is not desired [104].
Chapter 6: General Conclusions and Future Work
6. General Conclusions and Future Work

Collagen gels from rat tail had a higher mechanical strength than the bovine corium. Acid soluble based collagen gels had a stronger mechanical stiffness than pepsin soluble collagen due to the lack of telopeptides. The collagen source and lack of telopeptides did not affect the cell viability of HDFa cells. The lack of telopeptides inhibited contraction in cell embedded collagen gels after 24 h. Although cell embedded HA-T gels resulted in a significant decrease in cell viability, a higher cell seeding density and washing with media after crosslinking can be investigated as these approaches have shown higher cell viability levels \[68\].

A photochemically crosslinked hydrogel based vitreous substitute was successfully prepared which was clear and shear thinned in a similar manner to native vitreous. HA-T with a higher molecular weight HA could be investigated to further reduce the polymer concentration and potentially still allow for crosslinking to occur \[49\]. \textit{In-situ} photochemical crosslinking resulted in a significant decrease in cell viability of ARPE-19 cells so this crosslinked collagen-HA material may also be utilised in \textit{in-vitro} models such as the PK-Eye as a simulated vitreous fluid for preclinical pharmaceutical formulation research to develop intraocular medicines \[57\].

To more closely resemble the \textit{in-vivo} environment, an \textit{in-vitro} assay can be repeated by growing the ARPE-19 cells under a hydrogel (e.g. collagen) and then conducting the photochemical crosslinking process. Ascorbic acid acts as a free radical scavenger and can therefore reduce the amount of ROS generated. Ascorbic acid has been used in photochemical crosslinking of collagen \textit{in-vitro} to reduce the cytotoxicity of the reaction and may therefore be used in future work \[331\].

To avoid the need for chemical covalent conjugation reactions, an IPC preparation method was successfully optimised and a hydrogel with tuneable mechanical properties was prepared. The gel could be lyophilised successfully without affecting the mechanical and gelling properties of the IPC.

Cryo-TEM successfully imaged the resulting fibrillogensis of the collagen IPC. The imaging technique could be repeated with the inclusion of uranyl acetate stain to monitor the changes in the D-banding pattern of
collagen during fibrillogenesis [217]. Cryo-FIB-SEM showed an image of the internal morphology of the IPC gel in its hydrated state. The technique could be used to more precisely analyse the effect of collagen concentration on the pore size and the matrix structure of the collagen in the IPC gel.

Although, the inclusion of PEG and HA did not affect gelling the time, the effect of PEG and HA inclusion on the mechanical stiffness and mesh size of the IPC gel should be further analysed. The effect of inclusion of thermosensitive polymers can be also investigated. Such polymers may tune the gel properties further by reducing the mesh size and might allow the use of less collagen without affecting the gel morphology.

To avoid the need for using a hydroxyproline assay, the calorimetric BCA assay may be used to measure the collagen concentration by first using a collagenase enzyme to degrade the collagen in the IPC gel. The assay can then be conducted on the solution as the copper ions would have more access to the degraded collagen fibrils.

Powdered gel IPC formulations of ilomastat and AZ 8955 showed a slow release 14-day profile in a 100 µL depot with drug a loading of 6 mg/mL. Co-formulation of anti-scarring and anti-inflammatory agents such as corticosteroids (e.g. dexamethasone) may further improve the therapeutic efficacy of the formulation. As pirfenidone is clinically approved for IPF [237] it may be trialled orally before trichiasis surgery and for a certain period or after surgery to determine whether oral pirfenidone improves outcome. A stable eye drop formulation of pirfenidone could be also prepared for use in other anti-scarring conditions and inflammation such as Sjogren Syndrome [332]. An eyedrop form of ilomastat has been described [141], but there remain compliance issues for the use of eyedrops post trichiasis surgery. The AZ 8955 and ilomastat IPC formulations can be tested for other localise applications where MPP inhibition is therapeutically relevant including cancer [333] and lung fibrosis [334] for localised mediation of healing after surgery. The IPC may have potential to encapsulate other particulate or colloid associated formulations such as microbubbles, liposomes or micelles.

Drug losses occurred due to drug suspension transfer during the formulation process. This can be potentially be reduced by scaling up the volumes used during the mixing process. A FlakTeck Speed mixer may also
be used to prepare the formulation to reduce losses and avoid air bubble formation\textsuperscript{[265]}.

Electrosprayed pirfenidone PLGA particles did not show a sufficiently prolonged release profile as envisaged necessary for post trichiasis surgical wound management. Electrosprayed of core shell particles of PLGA-pirfenidone can be prepared to slow the pirfenidone drug release\textsuperscript{[335]}. Other polymers may also be evaluated to prepare the core shell particles including a more hydrophobic PLGA (e.g. 75:25 LA:GA ratio) to reduce the drug release further due increased polymer hydrophobicity, but reduced polymer degradation rate\textsuperscript{[254]} might become a foreign body response issue at the implant site. Due the high efficacy of both AZ 8955 and ilomastat, they may be prepared as PLGA core shell particles to slow drug release further. This can allow for applications in conditions were a slower release profile is required including lung fibrosis\textsuperscript{[334]}.

Not all the thermal transitions were fully separated when the IPC formulations and the PLGA-pirfenidone particles were analysed with the standard DSC. Modulated DSC should be used in the future to separate the thermal transition stages\textsuperscript{[282]}. DSC/modulated DSC should be used to analyse the IPC and its formulation in the liquid sate investigate whether the presence of EDTA, mannitol and sucrose increase the $T_d$ of collagen\textsuperscript{[247]}.

The collagen contraction assay showed that AZ 8955 was the most potent drug at inhibiting collagen contraction (ECM remodelling) followed by ilomastat and then pirfenidone. The contraction assay of HTF cells embedded in collagen gels should be carried out in higher concentrations of pirfenidone, e.g. 500–750 µg/mL where the drug does not affect the cell viability. The formulations should also be tested in collagen gels embedded with trichiasis affected HTF cells\textsuperscript{[141]} to study whether the AZ 8955 and ilomastat IPC formulation efficacy profile changes. The ilomastat and AZ 8955 IPC depots should be tested \textit{in-vivo} in the subconjunctiva in rabbits to test the pharmacokinetic profile of the formulations. The study would also identify whether the formulations prevent an immune response such as uptake of drug particles by macrophages or a foreign body response. Potentially, the difference in anti-scarring efficacy of the ilomastat and AZ 8955 IPC formulations could be studied by testing the effect of the formulations on bleb
survival after glaucoma filtration surgery in a rabbit model \cite{133}. The cell protective effect \cite{289} of ilomastat can be studied further in other cell lines where repair is required. An example can be corneal cells for corneal scarring, dermal fibroblasts for burn wounds or in nerve cells for peripheral nerve repair\cite{310}.

The IPC-HA formulation produced a slow release depot of bevacizumab which maintained ~80% of its anti-VEGF activity. Antibody degradation still occurred. The released samples need to be analysed further to establish what portion of the antibody aggregated and what portion is degraded and to what degree. The stability of bevacizumab may be improved by incorporation of excipients such as arginine, histidine and glutamate \cite{320}. Exploring other therapeutic proteins for preparing a slow release formulation of the IPC-HA hydrogel should be conducted.

A preparation method was established for the cell IPC formulation where the IPC can be mixed with cell suspension before delivery. The cell viability remained excellent even 7 days after incubation. The 3D cultures of HTF cells in the IPC gels can be stained with F-actin and anti-paxillin antibody on day 7 of the contraction assay experiment to test the effect of time on the cytoskeleton of the cells. Moreover, a range of 3D cultures in different concentrations of AS collagen should tested to establish what concentrations the collagen gel contraction is completely inhibited.

To further test the HTF-IPC formulation, its effect can be tested on a retinal explant cell viability and morphology. The IPC-cell preparation could also be injected subcutaneously into mice to test the cell survival and immune response \textit{in-vivo}. The IPC can also be formulated with other relevant cells such as adipose tissue stem cells to improve cell survival upon injection and to provide tissue support in tissue augmentation applications. The co-formulation of the IPC-cell system with growth factor proteins could be utilised as a vehicle to entrap cells to optimise the local concentration of appropriate growth factors.

Moreover, as described in \textbf{Chapter 2} the use of an LDH assay in future \textit{in-vitro} cellular studies could give a better picture of the levels of cellular cytotoxicity. An Apoptosis-Necrosis assay can be also be used in future work to analyse the type of cell death that occurs in any of those studies.
Exploring administration devices that allow the IPC and cell suspension or the IPC-drug cake (in the anti-scarring drugs or bevacizumab) and water to remain separate up to the point of delivery would be ideal. An example would be a dual chamber pre-fillable syringe which allows the mixing of two vehicles at the time of delivery [336].

In conclusion, this work has achieved its aim of developing a promising in-situ gelling anti-scarring formulation for the slow release of small drug molecules and proteins for clinical applications such as treating trichiasis.
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Appendix

Chapter 3

Figure 116. Hydroxyproline assay of type I collagen standard curve. \( y = 0.1533x + 0.0473 \), \( R^2 = 0.997 \). Values represent the mean ±SE at \( n=3 \).
Figure 117. The standard curve of ilomastat. \( n=3, y = 11647x + 958.96, R^2 = 1. \) Values represent the mean ±SE at \( n=3. \)

Figure 118. The standard curve of pirfenidone. \( n=3, y = 38811x + 15684, R^2 = 1. \) Values represent the mean ±SE at \( n=3. \)
Figure 119. The standard curve of AZ 8955. \( n=3, y = 31648x -50624, R^2 =0.9978. \) Values represent the mean ±SE at \( n=3. \)

Figure 120. The ilomastat HPLC peak at 280 nm. Retention= 6.6 min
**Figure 121.** The HPLC peak of pirfenidone at 265 nm. Retention time = 5.5 min.

**Figure 122.** The AZ8955 HPLC peak at 265 nm, retention time = 16.6 min.
Figure 123. The HPLC chromatogram peak of bevacizumab and collagen. a) bevacizumab, retention time = 2.152 min and b) collagen, retention time = 3.591 min.

Figure 124. The standard curve of bevacizumab. \( n=3, y = 5354.2x + 114.54 \), \( R^2 = 0.9993 \). Values represent the mean ±SE at \( n=3 \).